

MOLECULAR MECHANISMS OF HOST SPECIFIC VIRULENCE
AND AVIRULENCE CAUSED BY COTTON BLIGHT
AND CITRUS CANKER PATHOGENS

By

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TABLE OF CONTENTS

| | |
|--|-----|
| ACKNOWLEDGMENTS | ii |
| ABSTRACT | iv |
| CHAPTERS | |
| 1. INTRODUCTION | 1 |
| 2. HOST-SPECIFIC SYMPTOMS AND INCREASED RELEASE OF <i>XANTHOMONAS CITRI</i> AND <i>X. CAMPESTRIS</i> PV. MALVACEARUM FROM LEAVES ARE DETERMINED BY THE 102 BP TANDEM REPEATS OF <i>pthA</i> AND <i>avrb6</i> , RESPECTIVELY | 9 |
| 3. INACTIVATION OF MULTIPLE AVIRULENCE GENES IN <i>XANTHOMONAS CAMPESTRIS</i> PV. MALVACEARUM CREATED A NONPATHOGENIC ENDOPHYTE OF COTTON | 37 |
| 4. INTRAGENIC RECOMBINATION OF A SINGLE PLANT PATHOGEN GENE PROVIDES A MECHANISM FOR THE EVOLUTION OF NEW HOST SPECIFICITIES | 61 |
| 5. PLANT NUCLEAR TARGETING SIGNALS ENCODED BY A FAMILY OF <i>XANTHOMONAS</i> AVIRULENCE/PATHOGENICITY GENES | 87 |
| 6. SUMMARY AND CONCLUSIONS | 111 |
| LITERATURE CITED | 115 |
| BIOGRAPHICAL SKETCH | 129 |

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Several members of a *Xanthomonas* avirulence (*avr*) gene family were shown to play an important role in determining host-specific pathogenicity (*pth*) of cotton blight and citrus canker pathogens. *X. campestris* pv. *malvacearum* strain XcmH1005, the cotton blight pathogen, carries twelve members of what is now known as an *avr/pth* gene family. Six plasmid-borne members were previously shown to confer avirulence on cotton in a gene-for-genes manner. Four out of six newly cloned chromosomal members also conferred avirulence on cotton. To investigate possible pleiotropic functions of these *avr* genes in XcmH1005, marker exchange mutagenesis and complementation analyses were carried out. Gene *avrb6* was found to be a major *pth* gene of XcmH1005 and was required for severe pathogenic symptoms on cotton. Other *avr* genes in XcmH1005 also had an additive effect on pathogenicity. These *avr* genes did not contribute to bacterial

growth *in planta*, but were essential for the induction of host-specific symptoms and release of the pathogen onto the leaf surface, strongly indicating their role in pathogen dispersal. Another member of the same gene family, *pthA* of *X. citri*, was previously shown to be essential for symptoms of citrus canker disease and release of the pathogen to the leaf surface. By constructing chimeric genes among *pthA* and six other *avr/pth* genes, both avirulence and pathogenic specificities were found to be determined by the 102 bp tandem repeats present in the central region of all members of the gene family. Furthermore, intragenic recombination was demonstrated to provide a mechanism for the generation of new *avr/pth* genes with novel host specificities. Sequence analysis revealed that *pthA* and *avrb6* encoded proteins containing three nuclear localization sequences in the C-terminal region. The predicted PthA and Avrb6 proteins were constitutively expressed in *Xanthomonas* as detected by Western analysis using anti-PthA antibody. When transiently expressed in plant cells, β -glucuronidase fusion proteins containing the C-terminal region of PthA or Avrb6 were observed to localize specifically in the nucleus. A working hypothesis is proposed that Avr/Pth proteins enter the plant cell nucleus and directly affect gene expression, leading to the induction of host-specific symptoms.

CHAPTER 1 INTRODUCTION

Disease resistance of plants and virulence of microbial pathogens are governed by their combined genotypes. During the process of reciprocal evolution, many plant and microbial genes have coevolved to actively engage in pathogenic interactions between host and pathogen, thus determine the final outcome of plant diseases. Successful infection by microbial pathogens may require surface attachment, degradation of host chemical and physical barriers, production of toxins, and inactivation of plant defenses (Lamb *et al.* 1989). Overall, up to 100 genes may be required for bacterial pathogenesis (Panopoulos and Peet 1985, Daniels *et al.* 1988), including those involved in bacterial growth *in planta*, induction of pathogenic symptoms, host range and avirulence (Gabriel 1986). On the other hand, host plant resistance to infection involves preformed barriers, inactivation of microbial toxins, recognition of pathogen signals and induction of active plant defenses. In incompatible plant-bacteria interaction, induced resistance is usually associated with a hypersensitive response (HR), characterized by the rapid, localized death of plant cells at the site of infection. This process requires activation of plant resistance genes involving signal perception and transduction and plant defense genes involving biosynthesis of pathogenesis-related proteins, phytoalexins and other antibiotics (Dixon and Harrison 1990, Dixon and Lamb 1990).

Bacterial plant pathogens require virulence genes to infect and to colonize plants. A large group of genes, called *hypersensitive response* and *pathogenicity (hrp)* genes, have been found generally conserved in all phytopathogenic bacteria examined (*e. g.*, *Erwinia*, *Pseudomonas* and *Xanthomonas*) (Willis *et al.* 1991). Whatever the bacterial pathogens studied, typical *hrp*- mutants are completely nonpathogenic and elicit neither pathogenic symptoms on host plants nor hypersensitive response on nonhost plants. Most *hrp* genes are clustered on 20-30 kb DNA fragments and are conserved at the genus or family level. High levels of DNA homology are found among many *hrp* genes from different genera of phytobacteria (Boucher *et al.* 1992). Several biological functions are encoded by different *hrp* genes. Some *hrp* genes (*e.g.*, *hrpS* of *P. syringae* pv. *phaseolicola*) are clearly regulatory, responding to plant signals to induce transcription of *hrp* operon in a manner that may be analogous to the two-component signaling systems (Rahme *et al.* 1991). Other *hrp* genes are involved in the synthesis and export of pathogenicity factors that also induce an HR on nonhost plants. DNA sequence analysis reveals that several *hrp* genes of *Pseudomonas* and *Xanthomonas* are remarkably similar to key virulence genes of animal bacteria such as *Yersinia* and *Shigella*, which are involved in export of proteinaceous pathogenicity factors (Gough *et al.* 1992, 1993; Fenselau *et al.* 1992). Some *hrp* genes of *Erwinia* and *Pseudomonas* were shown to encode proteinaceous HR elicitors that are secreted via Hrp-dependent pathway (Wei *et al.* 1992; He *et al.* 1993). Since HR on host plants (race specificity) conferred by avirulence (*avr*) genes is dependent on *hrp* function, it was proposed that Hrp proteins

may also involve export of avirulence elicitors (Fenselau *et al.* 1992). Therefore, *hrp* genes may control or condition the functions encoded by virulence and avirulence genes.

Bacteria belonging to the genus *Xanthomonas* are plant-associated and infect 392 monocot and dicot species in 68 families (Leyns *et al.* 1984). Although the genus as a whole has wide host range, individual strains have host ranges limited to only certain plant species. This phenomenon of host specialization indicates that establishment of *Xanthomonas* infection involves host-specific recognition in addition to general virulence function of pathogen conferred by *hrp* genes. Host-specific virulence (*hsv*) genes encoding positive function have been shown to be a major determinant for host range and pathovar status of *Xanthomonas* (Gabriel *et al.* 1993). These genes are required for bacterial pathogens to grow and/or to induce pathogenic symptoms on specific host plants, but not in other plant species. Several host-specific virulence genes have been isolated from *X. campestris* pv. citrumelo (Kingsley *et al.* 1993), *X. c.* pv. translucens (Waney *et al.* 1992) and *X. citri* (Swarup *et al.* 1991). For example, *opsX* of *X. c.* pv. citrumelo is involved in biosynthesis of lipopolysaccharide and extracellular polysaccharide and is required for virulence on citrus, but not on bean (Kingsley *et al.* 1993). These *hsv* genes are analogous to host-specific nodulation (*hsn*) genes of *Rhizobium*. The *hsn* genes of *Rhizobium* is involved in host-specific modification of lipooligosaccharide Nod factors and function only for nodulation on specific hosts (Fisher and Long 1992; Denarie *et al.* 1992).

Besides *hsv* genes, *avr* genes have been shown to play a role in determining host specificity at the species level (Keen 1990; Heath 1991). Single cloned *avr* genes from

a pathogen of one host species can cause an otherwise virulent pathogen of another host species to become avirulent on its own host (Kobayashi *et al.* 1989; Whalen *et al.* 1988). Therefore, host species specificity is conditioned by both host-specific virulence and avirulence genes with the former as the major determinant.

Although *avr* genes may condition host range at the species level in some cases, they act as negative factors to confer avirulence and to limit host range only if the host plants have specific resistance (*R*) genes. The interaction of pathogens with *avr* genes and host plants with specific *R* genes results in the plant defense response, often known as hypersensitive response. This genetic requirement for specific plant *R* genes and specific microbial *avr* genes is called a gene-for-gene interaction. The gene-for-gene interactions were first demonstrated by Flor (1942, 1946, 1947) using flax and flax rust and have since been observed in many plant-parasite interactions between fungi, bacteria, virus, nematodes and their respective hosts (Thompson and Burdon 1992).

During the past decade, many *avr* genes have been isolated from different bacterial and fungal plant pathogens (Gabriel *et al.* 1993; Keen 1990). In some instances, *avr* genes encode proteinaceous elicitors that directly induce the HR on resistance plants. For example, *avr4* and *avr9* of the fungal pathogen *Cladosporium fulvum* encode race-specific peptides which are excreted into the apoplast of infected leaves and induce the HR on tomato cultivars that carry resistance genes *Cf4* and *Cf9*, respectively (Joosten *et al.* 1994; van Kan *et al.* 1991). In another case, *avrD* of *Pseudomonas syringae* pv. tomato appears to encode a enzyme involved in synthesis of a race-specific glycolipid elicitor (Keen *et al.* 1990; Kobayashi *et al.* 1990, Midland *et*

al. 1993). In most cases, however, the biochemical identities of *avr* gene products are unknown and the sequence analyses of *avr* genes are uninformative as to how they might interact with *R* gene products to trigger the HR and specify gene-for-gene interaction.

Several models (including elicitor/receptor model, dimer model and ion channel defense model) have been proposed to explain the molecular and biochemical bases of gene-for-gene recognition between plant and pathogen (Gabriel and Rolfe 1990). But very little experimental evidence is available to prove any of the hypotheses. Recently, the first plant *R* gene, *Pto*, has been isolated from tomato by map-based positional cloning (Martin *et al.* 1993). This gene encodes a serine/threonine protein kinase that is homologous to the receptor protein kinase SRK6 involving *Brassica* pollen-stigma recognition and to the Raf protein kinase involving the mammalian Ras signaling pathway. The Ras pathway is the best known circuit of signal transduction found in yeast, insect, as well as mammalian systems (Culotta and Koshland 1993). The Ras pathway begins with extracellular signals (*e. g.* growth factors or cytokines) that interact with receptor protein kinases, which in turn trigger phosphorylation cascades and finally expression of targeted genes (Crews and Erikson 1993).

Although Ras-like signaling pathway has not been demonstrated in plants, homologues of mammalian signal transduction components were found in many plants. Those include homologues of G-protein subunits (*e. g.* auxin-regulated ArcA of tobacco, Ishida *et al.* 1993), receptor protein kinases (*e.g.* pollen-specific SRK of *Brassica*, Goring and Rothstein 1992), Ras-related proteins (*e.g.* Rgpl of rice, Kamada *et al.* 1992), Raf (*e.g.*, Pto of tomato, Martin *et al.* 1993; and ethylene-regulated CTR1 of

Arabidopsis, Kieber *et al.* 1993) and mitogen-activated protein kinase (e.g., MsERK1 of alfalfa, Duerr *et al.* 1993). It is possible that virulence and avirulence gene products may activate the similar signal transduction pathway in plants, leading to disease symptoms or hypersensitive response. For example, transcriptional activation of plant defense genes is modulated by phosphorylation (Felix *et al.* 1991; Yu *et al.* 1993) and can be blocked by inhibitors of mammalian protein kinases (Raz and Fluhr 1993). It was proposed that recognition of *avr* signals by *R* gene products would trigger a phosphorylation cascade, leading to plant defense responses (Lamb 1994).

In this study, two economically important, world-wide diseases caused by *Xanthomonas* have been used as model systems. Cotton bacterial blight is caused by *X. campestris* pv. *malvacearum*; symptoms include angular, watersoaked spots on leaves, black arm on stems and bollrot (Verma 1986). Citrus canker is caused by *X. citri*; symptoms include erumpent, corky hyperplasia on leaves, stems and fruit (Schoulties *et al.* 1987; Stall and Civerolo 1991). Recently, members of a *Xanthomonas* avirulence (*avr*) and pathogenicity (*pth*) gene family were found to play an important role in determining host-specific virulence and avirulence caused by these two pathogens (De Feyter and Gabriel 1991a; De Feyter *et al.* 1993; Swarup *et al.* 1991, 1992). For example, a pathogenicity gene, *pthA*, is required for *X. citri* to elicit hyperplastic lesions on citrus (Swarup *et al.* 1991). It also functions as an *avr* gene to confer the ability to elicit an HR on cotton and bean (Swarup *et al.* 1992). Members of this gene family also include *avrB4*, *avrb6*, *avrb7*, *avrBln*, *avrB101* and *avrB102* of *X. campestris* pv. *malvacearum* (De Feyter and Gabriel 1991a; De Feyter *et al.* 1993), *avrBs3*, *avrBs3-2*

and *avrBsP* of *X. campestris* pv. *vesicatoria* (Bonas *et al.* 1989, 1993; Canteros *et al.* 1991), and *avrxa5*, *avrXa7* and *avrXa10* of *X. oryzae* (Hopkins *et al.* 1992). The most conspicuous feature of this gene family is the nearly identical, tandemly arranged, 102bp repeats in the central portion of genes (Fig. 1-1). Deletion analyses of *avrBs3* have shown that the 102bp repeated motifs determines the avirulence specificity of the gene (Herbers *et al.* 1992). However, many questions remain regarding to their pleiotropic functions, specificity, evolution, and biochemical mechanism of gene-for-gene recognition.

The primary objectives of this work were to determine (1) why avirulence genes are present in the cotton blight pathogen and whether they confer any pleiotropic functions, (2) what determines avirulence and pathogenic specificity in cotton blight and citrus canker pathogens, (3) how the specificity is created and evolved in this *avr/pth* gene family, and (4) how the signals encoded by the *avr/pth* genes are perceived and transduced in plant cells.




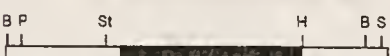
| Name | Gene Structure | # 102bp Repeats | Pleiotropic Function |
|----------------|--|-----------------|----------------------|
| <i>pthA</i> |  | 17.5 | cankering |
| <i>avrb6</i> |  | 13.5 | watersoaking |
| <i>avrBs3</i> |  | 17.5 | --- |
| <i>avrXa10</i> |  | 15.5 | --- |

Figure 1-1. A *Xanthomonas avr/pth* gene family. Listed are four representative members: *pthA* of *X. citri*, *avrb6* of *X. campestris* pv. *malvacearum*, *avrBs3* of *X. campestris* pv. *vesicatoria*, and *avrXa10* of *X. oryzae* pv. *oryzae*. The tandemly arranged blocks represent the 102-bp repeats in the central portion of genes. Restriction enzyme cleavage sites, relevant to this work and found in most members of the gene family, are: B, *Bam*HI; P, *Pst*I; St, *Stu*I; H, *Hinc*II; S, *Sst*I.

CHAPTER 2
HOST-SPECIFIC SYMPTOMS AND INCREASED RELEASE OF *XANTHOMONAS*
CITRI AND *X. CAMPESTRIS* PV. *MALVACEARUM* FROM LEAVES
ARE DETERMINED BY THE 102BP TANDEM REPEATS
OF *pthA* AND *avrb6*, RESPECTIVELY

Introduction

Microbial genes involved in plant-microbe interactions may be functionally classified into four broad categories: parasitic, pathogenic, host range and avirulence (Gabriel 1986). Genes involved in parasitism are absolutely required for growth *in planta* and are widely conserved at the family or genus level. Examples include most *hrp* (hypersensitive response and pathogenicity) genes of *Erwinia*, *Pseudomonas* and *Xanthomonas*, and the common *nod* (nodulation) genes of *Rhizobium* (Boucher *et al.* 1992, Denarie *et al.* 1992, Willis *et al.* 1991). Genes involved in pathogenicity are required for induction of symptoms. Examples include pectate lyase, polygalacturonase and endoglucanase genes (Collmer and Keen 1986, Schell *et al.* 1988, Roberts *et al.* 1988), *dsp* (disease specific, Arlat and Boucher 1991), *wts* (watersoaking, Coplin *et al.* 1992) genes, phytohormone biosynthetic genes (Smidt and Kosuge 1978) and toxin biosynthetic genes (Mitchell 1984). Genes involved in conditioning host range are host specific and required for growth on specific hosts. Examples include *hsv* (host-specific virulence), *pth* (pathogenicity), and *hsn* (host-specific nodulation) genes of *Pseudomonas*,

Xanthomonas and *Rhizobium* (Denarie *et al.* 1992, Kingsley *et al.* 1993, Ma *et al.* 1988, Salch and Shaw 1988, Swarup *et al.* 1991, Waney *et al.* 1991, Gabriel *et al.*, 1993). Information on conservation of these genes within species, pathovars and biovars is scarce, but these genes appear to determine biovar and pathovar status (Djordjevic *et al.*, 1987; Gabriel *et al.*, 1993). The fourth group are termed avirulence (*avr*) genes because they negatively affect virulence. The *avr* genes are superimposed on basic compatibility (Ellingboe 1976), are not highly conserved, and determine pathogenic races below the species, biovar or pathovar levels. These four broad categories are not mutually exclusive.

The interaction of microbes having *avr* genes and host plants having resistance (*R*) genes can result in plant defense responses, often observed visually as a hypersensitive reaction (HR) and characterized by the rapid necrosis of plant cells at the site of infection and the accumulation of phytoalexins. Most *avr* genes do not appear to confer selective advantage to the pathogen (Gabriel 1989, Keen and Staskawicz 1988). Pleiotropic functions have been identified for only 3 of the 30 *avr* genes cloned to date (Gabriel *et al.*, 1993). Furthermore, the DNA sequences of the cloned *avr* genes have been remarkably uninformative in terms of function (Keen, 1990). The presence of most *avr* genes in plant pathogens therefore remains enigmatic.

Recently, an *avr* gene family has been discovered in many different xanthomonads; members include *avrBs3*, *avrBs3-2* and *avrBsP* (Bonas *et al.* 1989, 1993; Canteros *et al.* 1991) of *X. campestris* pv. *vesicatoria*, *avrB4*, *avrb6*, *avrb7*, *avrBln*, *avrB101* and *avrB102* of *X. campestris* pv. *malvacearum* (De Feyter and Gabriel 1991a,

De Feyter *et al.* 1993) and *avrxa5*, *avrXa7* and *avrXa10* of *X. oryzae* (Hopkins *et al.* 1992). Interestingly, this gene family includes a gene, *pthA*, that is required for pathogenicity of *X. citri* on citrus. This gene is not known to function for avirulence in *X. citri* (Swarup *et al.* 1992), but is required for *X. citri* to induce cell divisions in the leaf mesophyll of citrus, leading to epidermal rupture and subsequent release of the bacteria onto the leaf surface. The gene also confers the ability to induce cell divisions on citrus to *X. campestris* strains from several different pathovars (Swarup *et al.* 1991, 1992).

De Feyter and Gabriel (1991a) observed that *avrb6* and *avrb7* enhanced the watersoaking ability of several *X. campestris* pv. *malvacearum* strains on cotton, but the role of these genes in pathogenicity was not determined. The family therefore consists primarily of *avr* genes, but includes at least one, and perhaps more, host-specific pathogenicity genes. The most conspicuous feature of this highly homologous gene family is the presence of nearly identical, tandemly arranged, 102bp repeats in the central region of the genes. These repeats are known to determine the gene-for-gene specificity of *avrBs3* (Herbers *et al.* 1992). The purpose of this study was to characterize the watersoaking functions of *avrb6* and to investigate the role of the 102bp repeats of *pthA*, *avrB4*, *avrb6*, *avrb7*, *avrBln*, *avrB101* and *avrB102* in pathogenicity and avirulence.

Materials and Methods

Bacterial strains, plasmids, and culture media. The bacterial strains and plasmids used in this study are listed in Table 2-1. Strains of *Escherichia coli* were grown in Luria-Bertani (LB) medium (Sambrook *et al.* 1989) at 37°C. Strains of *Xanthomonas* were grown in PYGM (peptone-yeast extract-glycerol-MOPS) medium at 30°C (De Feyter *et al.* 1990). For culture on solid medium, agar was added at 15g/L. Antibiotics were used at the following final concentrations (mg/L): ampicillin (Ap), 25; kanamycin (Km), 20; gentamycin (Gm), 3; spectinomycin (Spc), 50; tetracycline (Tc), 15; and rifampicin (Rif), 75.

Recombinant DNA techniques. Total DNA isolation from *Xanthomonas* was as described (Gabriel and De Feyter 1992). Plasmids were isolated from *E. coli* by alkaline lysis methods (Sambrook *et al.* 1989). Restriction enzyme digestion, alkaline phosphatase treatment, DNA ligation and random priming reactions were performed as recommended by the manufacturers. Southern hybridization was performed by using nylon membranes as described (Lazo *et al.* 1987). Otherwise, standard recombinant DNA procedures were used (Sambrook *et al.* 1989).

Construction of chimeric genes. To construct *Bam*HI fragment-swapped chimeric genes among *avrB4*, *avrb6*, *avrb7*, *avrBIn*, *avrB101*, *avrB102*, the *Bam*HI fragments from these genes were cloned into an *avrb7* shell (containing 5' and 3' ends of *avrb7*, but deleted for its *Bam*HI fragment, pUFR163Δ*Bam*) and an *avrBIn* shell (containing 5' and 3' ends of *avrBIn*, but deleted for its *Bam*HI fragment, pUFR186Δ*Bam*) on the

Table 2-1. Bacterial strains and plasmids used in this study.

| Strain or Plasmid | Relevant Characteristics | Reference or Source |
|---|---|-------------------------------|
| <i>E. coli</i> | | |
| DH5 α | F ⁻ , <i>endA1</i> , <i>hsdR17</i> (r _k ⁻ m _k ⁺), <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> , <i>gyrA</i> , <i>relA1</i> , ϕ 80 <i>dlacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169 | Gibco-BRL, Gaithersburg, MD |
| HB101 | <i>supE44</i> , <i>hsdS20</i> (r _k ⁻ m _k ⁺), <i>recA13</i> , <i>ara-14</i> , <i>proA2</i> , <i>lacY1</i> , <i>galK2</i> , <i>rpsL20</i> , <i>xyl-5</i> , <i>mil-1</i> | Boyer & Roulland-Dussoix 1969 |
| ED8767 | <i>supE44</i> , <i>supF58</i> , <i>hsdS3</i> (r _k ⁻ m _k ⁺), <i>recA56</i> , <i>galK2</i> , <i>galT22</i> , <i>metB1</i> | Murray <i>et al.</i> 1977 |
| <i>Xanthomonas citri</i> | | |
| 3213 | ATCC49118, citrus canker type strain | Gabriel <i>et al.</i> 1989 |
| B21.2 | <i>pthA::Tn5-gusA</i> , marker-exchange mutant of 3213 | Swarup <i>et al.</i> 1991 |
| <i>X. phaseoli</i> | | |
| G27 | ATCC49119, bean blight type strain | Gabriel <i>et al.</i> 1989 |
| G27Sp | Spc ^r derivative of G27 | Swarup <i>et al.</i> 1991 |
| <i>X. campestris</i> pv. <i>citrumelo</i> | | |
| 3048 | ATCC49120, citrus leaf spot pathotype strain | Gabriel <i>et al.</i> 1989 |
| 3048Sp | Spc ^r derivative of 3048 | Swarup <i>et al.</i> 1991 |
| <i>X. campestris</i> pv. <i>alfalfae</i> | | |
| KX-1Sp | Spc ^r derivative of KX-1, isolated from alfalfa, causing citrus leaf spot | Swarup <i>et al.</i> 1991 |
| <i>X. campestris</i> pv. <i>malvacearum</i> | | |
| XcmH | Natural isolate from cotton from Oklahoma; carries six <i>avr</i> genes used in this study on pXcmH, plus additional <i>avr</i> genes | De Feyter & Gabriel, 1991a |
| XcmH1005 | Spontaneous Rif ^r derivative of XcmH | This study |
| XcmH1407 | <i>avrb6::Tn5-gusA</i> , marker-exchange mutant of XcmH1005 | This study |
| XcmH1427 | <i>avrb7::Tn5-gusA</i> , marker-exchange mutant of XcmH1005 | This study |
| XcmH1431 | <i>avrBln::Tn5-gusA</i> , marker-exchange mutant of XcmH1005 | This study |
| XcmN | Natural isolate from cotton from Upper Volta, Africa | Gabriel <i>et al.</i> 1986 |
| Xcm1003 | Spc ^r Rif ^r derivative of XcmN | DeFeyter & Gabriel 1991a |
| Plasmid | | |
| pRK2013 | ColEI, Km ^r , Tra ⁺ , helper plasmid | Figurski & Helinski, 1979 |
| pRK2073 | pRK2013 derivative, npt::Tn7, Km ^r , Sp ^r , Tra ⁺ , helper plasmid | Leong <i>et al.</i> 1982 |
| pUFR042 | IncW, Km ^r , Gm ^r , Mob ⁺ , <i>lacZ</i> α ⁺ , Par ⁺ , | DeFeyter & Gabriel 1991a |
| pUFR047 | IncW, Gm ^r , Ap ^r , Mob ⁺ , <i>lacZ</i> α ⁺ , Par ⁺ | De Feyter <i>et al.</i> 1993 |

| Strain or Plasmid | Relevant Characteristics | Reference or Source |
|-------------------|---|------------------------------|
| pUFR049 | RSF1010 replicon, Cmr ^r , Sm ^r , IncW ⁺ , Mob ⁺ displacement vector | Swarup <i>et al.</i> 1991 |
| pUFR054 | IncP, Tc ^r , Mob ⁺ , containing methylases <i>Xma</i> I and <i>Xma</i> III | DeFeyter & Gabriel, 1991b |
| pUFR115 | 7.5kb fragment containing <i>avrB4</i> in pUFR042 | De Feyter & Gabriel 1991a |
| pUFR127 | 5kb fragment containing <i>avrb6</i> in pUFR042 | De Feyter & Gabriel 1991a |
| pUFR135 | <i>lacZ::avrb6</i> fusion in pUFR042 | De Feyter <i>et al.</i> 1993 |
| pUFR142 | 9kb fragment containing <i>avrB101</i> in pUFR047 | De Feyter <i>et al.</i> 1993 |
| pUFR156 | 12.9 kb fragment containing <i>avrBln</i> in pUFR042 | De Feyter <i>et al.</i> 1993 |
| pUFR157 | 11kb fragment containing <i>avrB102</i> in pUFR042 | De Feyter <i>et al.</i> 1993 |
| pUFR163 | 10kb fragment containing <i>avrb7</i> in pUFR042 | De Feyter <i>et al.</i> 1993 |
| pUFR163ΔBam | pUFR163 deleted for 3.4 kb <i>Bam</i> HI fragment | This study |
| pUFR171 | Internal <i>Bam</i> HI fragment of <i>avrB4</i> , in pGem11Zf(+) | De Feyter <i>et al.</i> 1993 |
| pUFR172 | Internal <i>Bam</i> HI fragment of <i>avrb6</i> , in pGem11Zf(+) | De Feyter <i>et al.</i> 1993 |
| pUFR173 | Internal <i>Bam</i> HI fragment of <i>avrB101</i> , in pGem11Zf(+) | De Feyter <i>et al.</i> 1993 |
| pUFR174 | Internal <i>Bam</i> HI fragment of <i>avrBln</i> , in pGem11Zf(+) | De Feyter <i>et al.</i> 1993 |
| pUFR175 | Internal <i>Bam</i> HI fragment of <i>avrB102</i> , in pGem11Zf(+) | De Feyter <i>et al.</i> 1993 |
| pUFR176 | Internal <i>Bam</i> HI fragment of <i>avrb7</i> , in pGem11Zf(+) | De Feyter <i>et al.</i> 1993 |
| pUFR178 | Internal <i>Bam</i> HI fragment of <i>avrBln</i> , in pGem11Zf(+), <i>Sal</i> I site filled | This study |
| pUFR179 | Internal <i>Bam</i> HI fragment of <i>avrb7</i> , in pGem11Zf(+), <i>Sal</i> I site filled | This study |
| pUFR180 | 9.5 kb fragment containing <i>avrb6</i> in pUFR042 | De Feyter <i>et al.</i> 1993 |
| pUFR186 | 10.3 kb <i>Bgl</i> II- <i>Eco</i> RI fragment containing <i>avrBln</i> from pUFR156 in pUFR047 | This study |
| pUFR186ΔBam | pUFR186 deleted for 3.7 kb <i>Bam</i> HI fragment | This study |
| pUFR190-194 | <i>Bam</i> HI-swapped chimeric genes with <i>avrb7</i> 5' and 3' ends plus <i>Bam</i> HI fragment of <i>avrB4</i> , <i>avrb6</i> , <i>avrBln</i> , <i>avrB101</i> , or <i>avrB102</i> in pUFR047 | This study |
| pUFR196-200 | <i>Bam</i> HI-swapped chimeric genes with <i>avrBln</i> 5' and 3' ends plus <i>Bam</i> HI fragment of <i>avrB4</i> , <i>avrb6</i> , <i>avrb7</i> , <i>avrB101</i> , or <i>avrB102</i> in pUFR047 | This study |
| pUFR205-209 | <i>Sna</i> I/ <i>Hinc</i> II-swapped chimeric genes with <i>avrBln</i> 5' and 3' regions plus <i>Sna</i> I/ <i>Hinc</i> II fragment of <i>avrB101</i> , <i>avrB4</i> , <i>avrb6</i> , <i>avrB102</i> , or <i>avrb7</i> in pUFR047 | This study |
| pUFR211-215 | <i>Sna</i> I/ <i>Hinc</i> II-swapped chimeric genes with <i>avrb7</i> 5' and 3' regions plus <i>Sna</i> I/ <i>Hinc</i> II fragment of <i>avrB101</i> , <i>avrB4</i> , <i>avrb6</i> , <i>avrBln</i> , or <i>avrB102</i> in pUFR047 | This study |
| pUFR217 | pUFR156 derivative, <i>avrBln::Tn5-gusA</i> | This study |
| pUFR220 | pUFR163 derivative, <i>avrb7::Tn5-gusA</i> | This study |
| pUFR227 | pUFR180 derivative, <i>avrb6::Tn5-gusA</i> | This study |
| pUFY019 | 3.7 kb <i>Sna</i> I/ <i>Hinc</i> II-swapped fragment with <i>pthA</i> 5' and 3' regions of pZit45 plus <i>avrb6</i> internal repeat region of pUFR135 in pUFR047 | This study |
| pUFY020 | 3.8 kb <i>Sna</i> I/ <i>Hinc</i> II-swapped fragment with <i>avrb6</i> 5' and 3' regions of pUFR135 plus <i>pthA</i> internal repeat region of pZit45 in pUFR047 | This study |
| pZit45 | 4.5kb fragment containing <i>pthA</i> in pUFR047 | Swarup <i>et al.</i> 1992 |
| pGEM7Zf(+) | ColE1, Ap ^r , <i>lacZa</i> ⁺ | Promega Co., Madison, WI |
| pGEM11Zf(+) | ColE1, Ap ^r , <i>lacZa</i> ⁺ | Promega Co., Madison, WI |

pUFR047 vector. The resulting *Bam*HI fragment-swapped chimeric genes formed pUFR190-200. The *avr* genes from pXcmH and *pthA* from *X. citri* all have unique *Stu*I and *Hinc*II sites delimiting the 102bp repeated regions, which allow the swapping of the internal repeated regions among these genes. To construct *Stu*I/*Hinc*II fragment-swapped chimeras using the pXcmH *avr* genes, pUFR174 and pUFR176 were cut with *Sal*I, blunt ended using the Klenow fragment and religated to destroy the *Hinc*II sites on the pGEM11Zf(+) portions of the plasmids, forming pUFR178 and pUFR179, respectively. These were then cut with *Stu*I and *Hinc*II to delete the internal fragments, and used as recipients for the *Stu*I/*Hinc*II internal fragments from all pXcmH *avr* genes on pUFR171-176. The *Bam*HI fragments from pUFR178 and its *Stu*I/*Hinc*II chimeras were recloned into pUFR186 Δ Bam, forming pUFR205-209. The *Bam*HI fragments from pUFR179 and its *Stu*I/*Hinc*II chimeras were recloned into pUFR163 Δ Bam, forming pUFR211-215. To facilitate construction of chimeric genes between *pthA* and *avrb6*, the 4.1 kb *Sal*I fragment containing *pthA* from pZit45 and the 3.0 kb *Eco*RI/*Sal*I fragment containing *avrb6* from pUFR135 were inserted into pGEM7Zf(+). The *Stu*I/*Hinc*II internal fragments were swapped between the two pGEM derivatives to create chimeric genes. The chimeric genes were then recloned as single *Eco*RI/*Hind*III fragments into pUFR047, forming pUFY019 and pUFY020.

Bacterial conjugation. Triparental matings were carried out to transfer broad host range plasmids from *E.coli* DH5 α to various *Xanthomonas* strain by using pRK2013 or pRK2073 as helper plasmids as described (De Feyter and Gabriel 1991a). To transfer

plasmids into Xcm, the modifier plasmid pUFR054 carrying *XcmI* and *XcmIII* methylase genes was used to increase the transfer frequency (De Feyter and Gabriel 1991b).

Marker-exchange mutagenesis. Marker-exchange mutagenesis of strain XcmH1005 was accomplished by introducing the displacement vector pUFR049 into XcmH1005 transconjugants harboring pUFR227 (*avrb6::Tn5-gusA*), pUFR220 (*avrb7::Tn5-gusA*) or pUFR217 (*avrBIn::Tn5-gusA*) derivatives. The procedure was the same as described (Swarup *et al.* 1991), except that the modifier plasmid pUFR054 was used to facilitate plasmid transfer into XcmH1005.

Plant inoculations. Cotton (*Gossypium hirsutum* L.) lines used were Acala-44 (Ac44) and its congeneric resistance lines B1, B2, B4, b6, b7, BIn and BIn3 as described (Swarup *et al.* 1992, De Feyter *et al.* 1993). Cotton plants were grown in the greenhouse, transferred to growth chambers before inoculation, and maintained under conditions as described (De Feyter and Gabriel 1991a). Bacterial suspensions of *X. campestris* pv. *malvacearum* (10^8 cfu/ml) in sterile tap water were gently pressure infiltrated into leaves of 4-5 week old cotton plants. Pathogenic symptoms were observed periodically 2-7 days after inoculation.

All citrus (*Citrus paradisi* 'Duncan', grapefruit) and bean (*Phaseolus vulgaris* 'California Light Red') plants were grown under greenhouse conditions. Plant inoculations involving *X. citri* or *pthA* or derivatives of *pthA* were carried out in BL-3P level containment (refer to Federal Register Vol.52, No.154, 1987) at the Division of Plant Industry, Florida Department of Agriculture, Gainesville. Bacterial suspensions were standardized in sterile tap water to 10^8 cfu/ml and pressure infiltrated into the

abaxial leaf surface of the plants. All plant inoculations on cotton, citrus or bean were repeated at least three times.

Bacterial growth in planta. To determine the growth of *X. campestris* pv. *malvacearum* in the susceptible cotton line Ac44, bacterial suspensions were adjusted to 10^6 cfu/ml and inoculated into cotton leaves by pressure infiltration. Leaf discs (1 cm^2) were taken by using a sterilized cork borer, then macerated in 1 ml sterile tap water. At least three samples were taken at each time point for each strain inoculated. Viable counts were determined by serial dilutions on plates containing appropriate antibiotics. Data shown in Fig. 2-2 are the mean and standard error from three experiments.

To quantify the amount of bacteria present on the surface of watersoaked leaf spots, leaves were inoculated with 10^7 cfu/ml bacterial suspensions from overnight cultures. Five days later, 100 μ l sterile tap water was dispensed onto the watersoaked leaf surface and spread to an area of approximately 1 cm^2 . After mixing with the bacteria and slime on the leaf surface, the bacterial suspension was collected with a pipet. Each 1 cm^2 watersoaked leaf area was washed ten times, and a total of 1 ml of bacterial suspension was collected. To quantify the bacteria remaining inside the leaf, leaf discs (1 cm^2) were taken with a sterilized cork borer, then macerated in 1 ml sterile tap water. Viable counts were determined by serial dilutions on plates containing appropriate antibiotics. Data reported in the results (external and total counts) were mean and standard error of six replicates from two experiments.

Results

Pleiotropic pathogenicity functions of *avrB6*. *X. campestris* pv. *malvacearum* strain XcmH carries *avrB4*, *avrB6*, *avrB7*, *avrBln*, *avrB101* and *avrB102* on a single plasmid, pXcmH, and elicits an HR on cotton lines carrying any one of many different resistance (*R*) genes (De Feyter *et al.*, 1993). Strain XcmH and a spontaneous rifamycin-resistant derivative of XcmH, XcmH1005, were virulent on susceptible cotton line Acala-44 (Ac44), and both elicited severe watersoaking and necrosis associated with growth *in planta* (XcmH1005 on Ac44 is shown in Figs. 2-1 & 2-2). Mutations of *avrB6*, *avrB7* and *avrBln* were individually generated in XcmH1005 by marker-exchange mutagenesis and each mutant was confirmed to carry a single Tn5-*gusA* insertion in the appropriate DNA fragment by Southern blot hybridization (refer Fig. 2-3; some data not shown). As predicted by gene-for-gene theory, marker-exchange mutants XcmH1407 (*avrB6*::Tn5-*gusA*), XcmH1427 (*avrB7*::Tn5-*gusA*) and XcmH1431 (*avrBln*::Tn5-*gusA*) gained virulence on cotton lines with the resistance genes *b6*, *b7* and *Bln*, respectively (Table 2-2). Plasmids pUFR127 (*avrB6*⁺), pUFR163 (*avrB7*⁺) and pUFR156 (*avrBln*⁺) were able to fully complement the specific avirulence defects of XcmH1407, XcmH1427 and XcmH1431, respectively.

Neither XcmH1427 nor XcmH1431 showed any change in watersoaking ability as compared with XcmH1005. However, marker-exchange mutant XcmH1407 not only lost avirulence on a cotton line with the *b6* resistance gene, but also elicited significantly less watersoaking and necrosis on susceptible cotton line Ac44 (Table 2-2 and Fig. 2-1A

Figure 2-1. Watersoaked lesions caused by *X. campestris* pv. *malvacearum* strains on the susceptible cotton line Acala-44. A. Leaf inoculated five days previously with: 1, XcmH1005; 2, XcmH1407; 3, XcmH1407/pUFR127; 4, XcmH1431. B. Leaf inoculated seven days previously with: 1, XcmH1005; 2, XcmH1407. C. Leaf inoculated five days previously with: 1, XcmH1005; 2, Xcm1003/pUFR127; 3, Xcm1003/pUFR156; 4, Xcm1003.

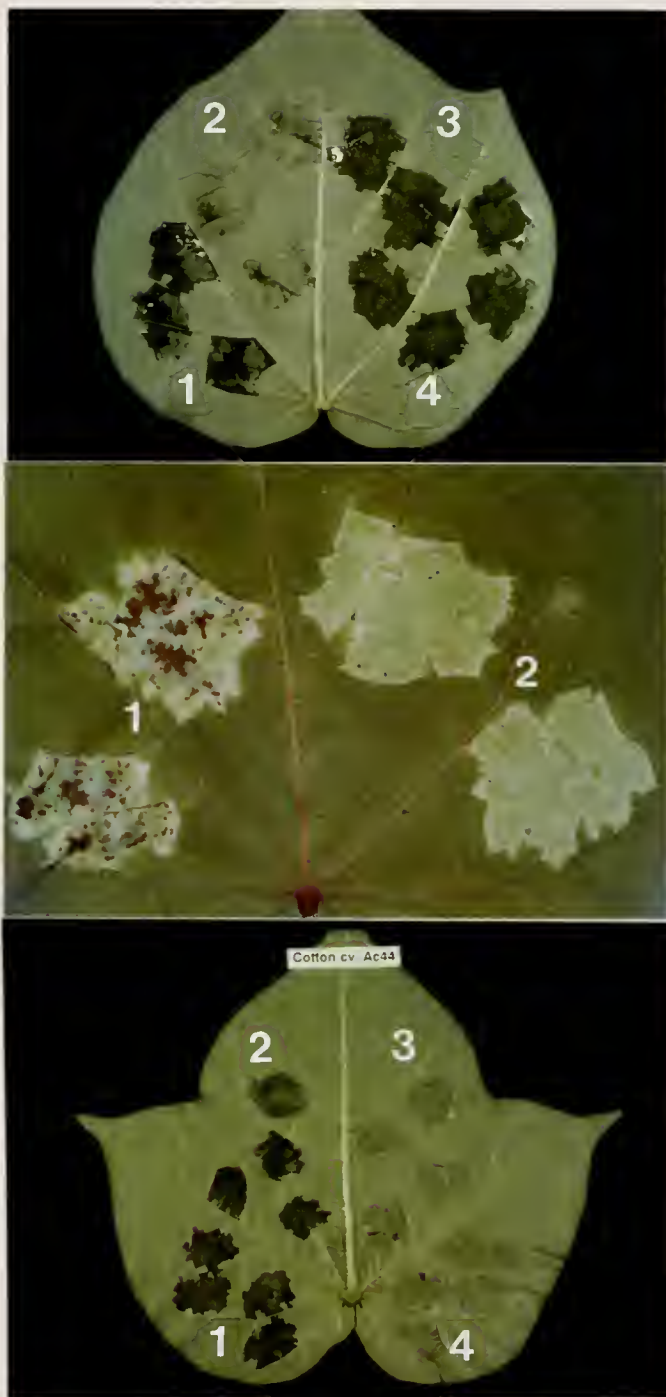
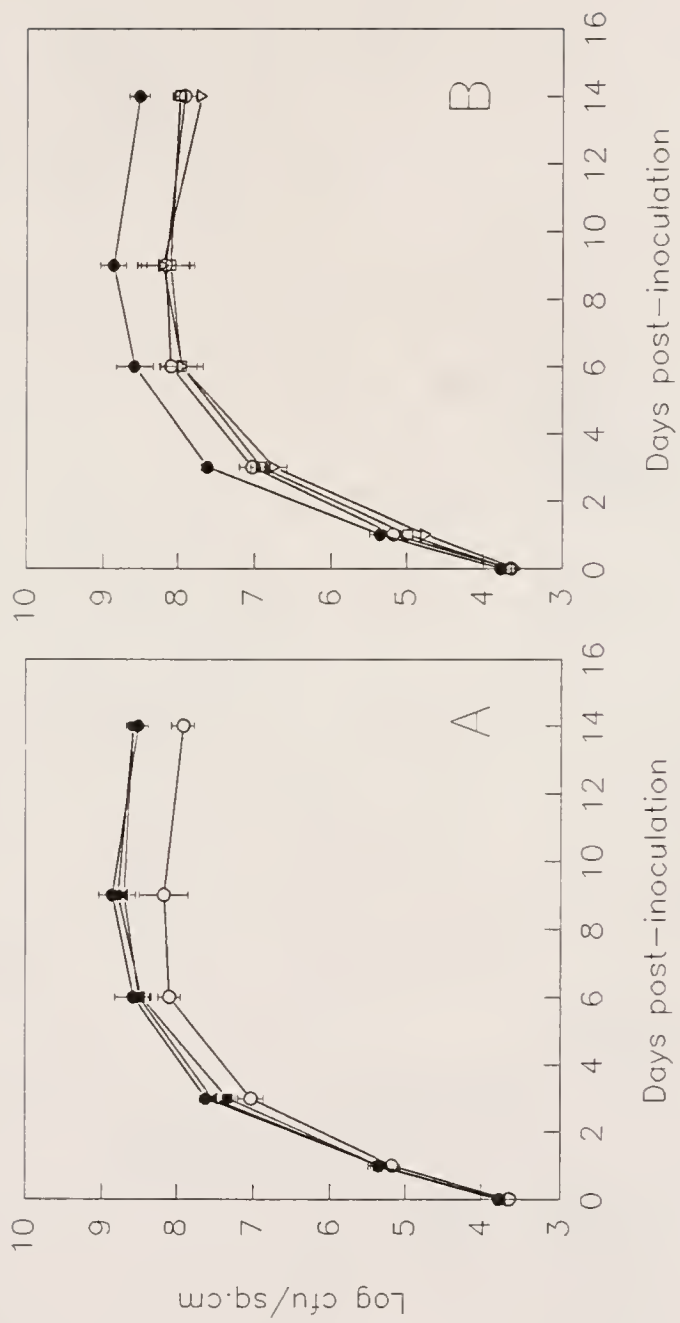


Figure 2-2. Growth kinetics of *X. campestris* pv. *malvacearum* strains on the susceptible cotton line Acala-44.
A. ● XcmH1005; ▲ XcmH1407; ■ XcmH1431; ○ Xcm1003. B. ● XcmH1005; ▽ Xcm1003/pUFR127;
Xcm1003/pUFR156; ○ Xcm1003.



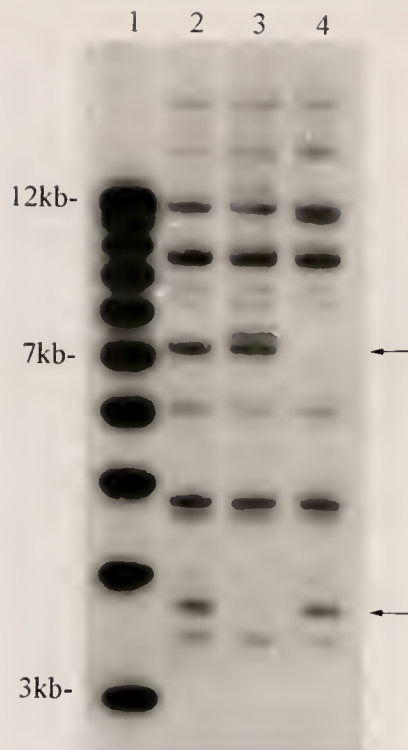


Figure 2-3. Southern blot hybridization of total DNA from XcmH1005 and marker-exchanged mutants of XcmH1005 after *EcoRI/SstI* digestion. The blot was probed with the internal 2.9kb *Bam*HI fragment from *avrb6*. The expected positions of fragments carrying each plasmid-borne member of the *avr/pth* gene family were determined from the restriction map of pXcmH [De Feyter and Gabriel, 1991]. The upper arrow indicates the expected position of the DNA fragment carrying *avrBIN*; the lower arrow indicates the position of *avrb6*. Lane 1, 1 kb DNA ladder; Lane 2, XcmH1005; Lane 3, XcmH1407; Lane 4, XcmH1431.

Table 2-2. Phenotypes of marker-exchange mutants of *Xanthomonas campestris* pv. *malvacearum* strain XcmH1005 on cotton cultivar Acala-44 (Ac44) and congenic lines^a.

| Xcm strains | Ac44 | Ac6 | Ac7 | AcBIn |
|--------------------------------------|------|-----|-----|-------|
| XcmH1005 | ++ | - | - | - |
| XcmH1407 (<i>avrb6::Tn5-gusA</i>) | + | + | - | - |
| XcmH1427 (<i>avrb7::Tn5-gusA</i>) | ++ | - | ++ | - |
| XcmH1431 (<i>avrBIn::Tn5-gusA</i>) | ++ | - | - | ++ |

^aAc6, Ac7, and AcBIn are congenic lines of Ac44 containing resistance gene *b6*, *b7*, and *BIn*, respectively. ++ = strong watersoaking symptoms; + = weak watersoaking symptoms; - = hypersensitive response.

& 2-1B). Plasmid pUFR127 was able to fully complement the pathogenicity defect(s) of XcmH1407 on susceptible cotton plants, in addition to complementing the specific avirulence defect. Despite its reduced ability to elicit pathogenic symptoms on susceptible cotton lines, mutant XcmH1407 exhibited the same growth rate and yield as that of the wild type XcmH1005 and XcmH1431 on Ac44 (Fig. 2-2A).

X. campestris pv. *malvacearum* strain Xcm1003 carries no known *avr* genes (De Feyter and Gabriel 1991a), caused less watersoaking on Ac44 than XcmH1005 and exhibited a lower growth rate on Ac44 than XcmH1005. Introduction of pUFR127 (*avrb6*⁺) into Xcm1003 conferred increased watersoaking ability (Fig. 2-1C), but growth rate and yield *in planta* of the transconjugant containing pUFR127 were not increased in comparison with that of Xcm1003 or the transconjugant Xcm1003/pUFR156 (*avrBl*⁺) (Fig. 2-2B). Therefore pUFR127 affected pathogenic symptoms in both XcmH1005 and Xcm1003, but not bacterial growth rate or yield *in planta* of either strain.

Strains containing *avrb6* (e.g. XcmH1005 and Xcm1003/pUFR127) elicited more severe watersoaking and necrosis and were associated with much more slime oozing from the watersoaked areas in comparison with strains lacking *avrb6* (e.g. XcmH1407 and Xcm1003; Fig. 2-1). The peak number of total colony forming units (cfu) per square centimeter of watersoaked leaf inoculated with XcmH1005 or XcmH1407 were basically the same ($1.41 \pm 0.09 \times 10^9$ cfu/cm² vs. $1.48 \pm 0.17 \times 10^9$ cfu/cm²). However, 14.1% of the XcmH1005 bacteria present in the lesion ($2.32 \pm 0.56 \times 10^8$ cfu/cm²) were released onto the surface of the leaf, whereas only 0.06% of the XcmH1407 bacteria ($9.64 \pm 4.42 \times 10^5$ cfu/cm²) were released onto the surface of the leaf.

Therefore, more than 240 times more bacteria were present on the external surface of the watersoaked lesions caused by XcmH1005 than by XcmH1407. In experiments where the leaves were moistened periodically after inoculation with a hand-held mist sprayer, *X. campestris* pv. *malvacearum* strains carrying *avrb6* always exhibited many secondary infections around the original inoculation site. In contrast, those strains lacking *avrb6* rarely exhibited secondary infections.

The pathogenicity functions encoded by *pthA* and *avrb6* are host specific and determined by 102bp repeats. Single *StuI* and *HincII* sites are found in *avrb6* at positions 1281 and 2860, respectively, that closely flank the 102 bp tandemly repeated region of the gene (De Feyter *et al.* 1993). Unique *StuI* and *HincII* sites are also found at the same relative positions in *pthA*, and the DNA sequences of *avrb6* and *pthA* are identical in the flanking regions from the 102 bp tandem repeats to these restriction sites (refer to Chapter 4). Chimeric genes were constructed by swapping the *StuI/HincII* fragments containing the internal 102bp repeated regions between *pthA* and *avrb6*. At least three individual clones of both chimeric genes were introduced into a mutant *X. citri* strain B21.2 (*pthA*::Tn5-*gusA*) and wild type strains of the following pathogens: *X. phaseoli* strain G27 (host range on bean), *X. campestris* pv. *citrumelo* strain 3048 (host range on bean and citrus), *X. campestris* pv. *alfalfae* strain KX-1 (host range on alfalfa, bean and citrus) and Xcm1003. Strains and transconjugants were inoculated on citrus, bean, and cotton leaves. As shown in Figure 2-4, pUFY020, carrying a chimeric gene containing 5' and 3' ends of *avrb6* and the internal repeats of *pthA*, complemented the mutant B21.2 to full virulence on citrus. The citrus canker symptoms caused by B21.2/pUFY020 were



Figure 2-4. Phenotypes of *X. citri* wild type and mutant strains on a grapefruit (*Citrus paradisi* cv. 'Duncan') leaf. 1, wild-type strain 3213; 2, marker-exchange mutant of 3213, B21.2; 3, B21.2/pZit45; 4, B21.2/pUFY020; 5, B21.2/pUFR135; 6, B21.2/pUFY019.

indistinguishable from the symptoms caused by B21.2/pZit45 (*pthA*⁺). When pUFY020 was present in 3048 or KX-1, both of which cause watersoaked leaf spots on citrus, canker symptoms were induced by the transconjugants that were indistinguishable from those conferred by pZit45 in the same strains. Also like pZit45 (Swarup *et al.*, 1992), pUFY020 conferred avirulence on bean to 3048, KX-1 and G27, and on cotton to Xcm1003.

A chimeric gene containing the 5' and 3' ends of *pthA* and the internal repeats of *avrb6* on pUFY019 enhanced the ability of Xcm1003 and XcmH1407 to watersoak cotton and cause necrosis. The watersoaking symptoms caused by Xcm1003/pUFY019 were indistinguishable from those caused by Xcm1003/pUFR127. This chimeric gene on pUFY019 behaved like *avrb6* on pUFR127 and conferred no detectable symptoms to B21.2, 3048 or G27 on citrus or bean.

Cultivar-specific avirulence is determined by 102bp repeats. Each of the seven *avr/pth* genes (*avrB4*, *avrb6*, *avrb7*, *avrBln*, *avrB101*, *avrB102*, and *pthA*) exhibits unique avirulence specificity in Xcm1003 on cotton resistance lines differing by single *R* genes (De Feyter *et al.* 1993, Swarup *et al.* 1992). Each of these seven genes contains two *Bam*HI sites, one near the 5' end and one near the 3' end of each gene, and single *Stu*I and *Hinc*II sites flanking the 102 bp direct repeat region (De Feyter *et al.* 1993, Swarup *et al.* 1992). To localize the region that determines the specificity of the reactions, a series of chimeric genes were constructed by swapping *Bam*HI and *Stu*I/*Hinc*II internal fragments among these seven members of the gene family. One to three individual clones of each chimeric gene were introduced into Xcm1003 and

Table 2-3. Avirulence specificity of *Bam*HI-fragment swapped chimeric genes in Xcm1003 on cotton.

| Plasmid | Chimeric Gene | | Avirulence Specificity |
|---------|---------------|------------------------|------------------------|
| | 5' and 3' end | <i>Bam</i> HI fragment | |
| pUFR190 | <i>avrb7</i> | <i>avrB4</i> | <i>avrB4</i> |
| pUFR191 | <i>avrb7</i> | <i>avrb6</i> | <i>avrb6</i> |
| pUFR192 | <i>avrb7</i> | <i>avrBIn</i> | <i>avrBIn</i> |
| pUFR193 | <i>avrb7</i> | <i>avrB101</i> | <i>avrB101</i> |
| pUFR194 | <i>avrb7</i> | <i>avrB102</i> | <i>avrB102</i> |
| pUFR196 | <i>avrBIn</i> | <i>avrB4</i> | <i>avrB4</i> |
| pUFR197 | <i>avrBIn</i> | <i>avrb6</i> | <i>avrb6</i> |
| pUFR198 | <i>avrBIn</i> | <i>avrb7</i> | <i>avrb7</i> |
| pUFR199 | <i>avrBIn</i> | <i>avrB101</i> | <i>avrB101</i> |
| pUFR200 | <i>avrBIn</i> | <i>avrB102</i> | <i>avrB102</i> |

Note: Chimeric genes were introduced into Xcm1003 and tested on cotton cv. Ac44 and its congenic resistance lines AcB1, AcB2, AcB4, Acb6, Acb7, AcBIn and AcBIn3 for avirulence specificity.

Table 2-4. Avirulence specificity of *StuI/HincII*-fragment swapped chimeric genes in Xcm1003 on cotton.

| Plasmid | Chimeric Gene | | Avirulence Specificity |
|---------|--------------------|-----------------------------|------------------------|
| | 5' and 3' region | <i>StuI/HincII</i> fragment | |
| pUFR205 | <i>avrBln</i> | <i>avrB4</i> | <i>avrB4*</i> |
| pUFR206 | <i>avrBln</i> | <i>avrb6</i> | <i>avrb6*</i> |
| pUFR207 | <i>avrBln</i> | <i>avrb7</i> | <i>avrb7*</i> |
| pUFR208 | <i>avrBln</i> | <i>avrB101</i> | <i>avrB101</i> |
| pUFR209 | <i>avrBln</i> | <i>avrB102</i> | <i>avrB102</i> |
| pUFR211 | <i>avrb7</i> | <i>avrB4</i> | <i>avrB4</i> |
| pUFR212 | <i>avrb7</i> | <i>avrb6</i> | <i>avrb6</i> |
| pUFR213 | <i>avrb7</i> | <i>avrBln</i> | <i>avrBln*</i> |
| pUFR214 | <i>avrb7</i> | <i>avrB101</i> | <i>avrB101</i> |
| pUFR215 | <i>avrb7</i> | <i>avrB102</i> | ND |
| pUFY019 | <i>pthA</i> | <i>avrb6</i> | <i>avrb6</i> |
| pUFY020 | <i>lacZ::avrb6</i> | <i>pthA</i> | <i>pthA</i> + |

Note: Chimeric genes were introduced into Xcm1003 and tested on cotton cv. Ac44 and its congenic resistance lines AcB1, AcB2, AcB4, Acb6, Acb7, AcBln and AcBln3 for avirulence specificity. * = weak avirulence; + = strong avirulence; ND = no avirulence detected.

inoculated on cotton cultivar Ac44 and its congenic resistance lines, each differing by a single *R* gene (*B1*, *B2*, *B4*, *b6*, *b7*, *BIn* or *BIn3*). The avirulence specificities of the seven *avr/pth* genes were first localized within the internal *Bam*HI fragments (Table 2-3), and then were further localized within the internal *Stu*I/*Hinc*II fragments (Table 2-4). In all cases, the avirulence specificity of a given gene was determined inside the *Stu*I/*Hinc*II (tandem repeat) region.

Discussion

Plant pathologists have long been puzzled by the presence of avirulence genes in pathogens. These genes act as negative factors to limit virulence and in most cases do not appear to provide selective advantage to the pathogens (Ellingboe, 1976; Gabriel, 1989; Keen and Staskawicz 1988). Rare exceptions have been reported. For example, *avrBs2* from *X. campestris* pv. *vesicatoria* is required for optimal growth *in planta* (Kearney and Staskawicz 1990). Both *avrb6* and *avrb7* from *X. campestris* pv. *malvacearum* strain XcmH are known to enhance the watersoaking ability of *X. campestris* pv. *malvacearum* strain Xcm1003 on cotton (De Feyter and Gabriel 1991a). In this study we demonstrated that the ability of strain XcmH1005 to cause strong watersoaking and necrosis on cotton requires the presence of *avrb6* but not *avrb7*. Although *avrb6* increased symptom elicitation by both Xcm1003 and XcmH1005, neither their growth rates nor their maximum bacterial counts per square centimeter of leaf were affected by the presence or absence of *avrb6*. Therefore *avrb6* functions as a

pathogenicity gene and increases symptoms of cotton blight, but without eliciting an HR on cotton lines lacking *b6*.

The strong watersoaking ability conferred by *avrb6* was correlated with much higher (240-fold) levels of bacterial cells released from inside the plant leaf to the surface. Since bacterial blight of cotton is usually spread by rain splash, the presence of large numbers of bacteria on the leaf surface would undoubtedly contribute to the dissemination of the population. Strains carrying *avrb6* would thereby have a selective advantage on cotton plants lacking the *b6* gene. Similarly, *pthA* appears to aid in the dissemination of *X. citri* by rupturing leaf epidermis and releasing bacteria, although it does so by inducing tissue hyperplasia (Swarup *et al.* 1991). Therefore, *pthA* and *avrb6* not only contribute to the amount of damage caused by their respective xanthomonads to their hosts, but may also contribute to the ecological fitness of their respective bacterial populations as pathogenicity genes.

Both *pthA* and *avrb6* may help determine host range in a positive manner, and not as *avr* genes. When *pthA* was transferred to *X. campestris* pv. *malvacearum* Xcm1003 and *X. phaseoli* G27, it conferred avirulence on the respective hosts, and did not induce tissue hyperplasia (Swarup *et al.*, 1992). In the present study, when *avrb6* was transferred to *X. citri* B21.2, *X. campestris* pv. *citrumelo* 3048 and *X. phaseoli* G27, it conferred no detectable effect when these strains were inoculated onto their respective hosts. Therefore, the pathogenicity functions of *avrb6* and *pthA* are host specific. If release of the pathogen to the leaf surface is host specific and it contributes to the ecological fitness of the pathogen as we propose, then *avrb6* and *pthA* function to

determine host range. Furthermore, the avirulence conferred by *pthA* appeared to be gratuitous in terms of restricting host range (Swarup *et al.*, 1992) and in the present study, *avrb6* failed to confer avirulence to three other pathogens. Therefore if *avrb6* and *pthA* help determine host range, it is not because of their function as *avr* genes. In a formal genetic sense, these pleiotropic *avr/pth* genes resemble some *Rhizobium* host-specific nodulation (*hsn*) genes which are required for host range on some hosts, but which can also confer avirulence when transferred to other *Rhizobium* strains with a different host range (Debelle *et al.* 1988; Faucher *et al.* 1989; Lewis-Henderson and Djordjevic 1991).

By swapping the tandemly repeated regions, the avirulence specificities of *avrB4*, *avrb6*, *avrb7*, *avrBln*, *avrB101*, *avrB102* and *pthA* were shown to be determined by the 102bp tandem repeats. These results are consistent with and extend the findings of Herbers *et al.* (1992), who showed that the avirulence specificity of *avrBs3* is determined by the 102bp repeats of that gene. In addition, the swapping experiments clearly demonstrated that the pathogenicity functions of *pthA* and *avrb6* were distinct (cankers vs. watersoaking), host specific, and in both cases the pathogenic specificity was determined by their 102bp tandemly repeated regions. Furthermore, the use of chimeric genes also ruled out the possibility that the host-specific pathogenicity on cotton and citrus were result of additional, unidentified pathogenicity genes encoded on the plasmids used (pUFR127 and pZit45).

Extensive deletion analyses of both the 5' and 3' ends of the pXcmH *avr* genes showed that all sections of these genes are required for avirulence activity (De Feyter *et*

al. 1993). From the present study, all sections of *pthA* and *avrb6* were required to confer pathogenicity functions. However, the 5' and 3' ends of the genes outside of the repeats appeared to be isofunctional among members of the gene family.

Besides the members of this *Xanthomonas avr/pth* gene family, internal tandem repeats are found in some pathogenicity genes of animal pathogens. Examples include the outer membrane protein A gene (*ompA*) of some *Rickettsia* species (Anderson *et al.* 1990, Gilmore 1993), internalin gene (*inl*) of *Listeria monocytogenes* (Gaillard *et al.* 1991), toxin A gene of *Clostridium difficile* (Dove *et al.* 1990), and M protein genes (*emm*) of *Streptococcus* (Hollingshead *et al.* 1987). Tandem repeats were also found in many genes from protozoan and metazoan parasites such as *Plasmodium* (McConkey *et al.* 1990), *Trypanosoma* (Hoft *et al.* 1989), *Leishmania* (Wallis and McMaster 1987), and *Meloidogyne* (Okimoto *et al.* 1991). Genes from *Trypanosoma cruzi* (the protozoan agent of American trypanosomiasis) and mitochondria of *Meloidogyne javanica* (plant root knot nematode) contain 102 bp tandem repeats. Most of these genes encode surface proteins and the distinctive arrangement of the tandem repeats in these genes are thought to encode a protective, strain-specific conformational epitope for evasion of host immunity (Gilmore 1993, Hoft *et al.* 1989, McConkey *et al.* 1990). By contrast, AvrBs3 is mainly located in cytosol (Knoop *et al.* 1991, Brown *et al.* 1993) and it is not clear how it may interact with the plant cell.

Southern hybridization has shown that potential members of the *avr/pth* gene family exist, often in multiple copies, in 9 of 12 *Xanthomonas* species or pathovars examined (Bonas *et al.* 1989, Swarup *et al.* 1992, De Feyter *et al.* 1993). In all *X. citri*

and *X. campestris* pv. *malvacearum* cotton strains tested to date, multiple DNA fragments hybridizing to *pthA* and *avrb6* are found. The pleiotropic pathogenicity functions of *pthA* and *avrb6* and their potential fitness value may explain why these genes are maintained in plant pathogens. Based on these data and knowledge of the functions of *avrb6* and *pthA*, we assume that all strains of *X. campestris* pv. *malvacearum* capable of strongly watersoaking cotton carry an *avrb6* gene or homologue that functions for pathogenicity. Similarly, we assume that all strains of *X. citri* capable of causing cankers on citrus carry a *pthA* gene. A number of pathogenic strains and pathovars in the genus *Xanthomonas* do not carry members of the gene family, and therefore these genes are not required for *Xanthomonas* virulence generally. Within pathovars where members of the *avr/pth* gene family are found in some, but not all strains tested (such as *X. campestris* pv. *vesicatoria*), there may be no pleiotropic pathogenicity function; for example, there is no evidence of a pathogenicity function of *avrBs3*, *avrBs3-2* and *avrBsP* (Bonas *et al.* 1989, 1993; Canteros *et al.* 1991).

The mechanism(s) by which leaf spotting pathogens elicit watersoaking and necrosis is unknown, but appears to involve both damage to leaf cell membranes (without eliciting an HR) and production of extracellular polysaccharide (EPS). The EPS does not appear to be involved in suppressing a potential HR, since production levels of EPS by *X. campestris* pv. *malvacearum* are similar in both susceptible and resistant cotton lines (Pierce *et al.* 1993). Instead, mutational analyses of *Xanthomonas* EPS biosynthetic genes and inoculations with purified EPS have shown that the EPS contributes to watersoaking by trapping water and nutrients in the intercellular spaces after they are

released (reviewed by Leigh and Coplin 1992). Coplin *et al.* (1992) have proposed that watersoaking and pathogenicity of *Erwinia stewartii* involves the EPS plus a cell leakage factor encoded by *wts* (watersoaking) genes. This hypothesis may well apply to *X. campestris* pv. *malvacearum*, with *avrb6* encoding a cell leakage factor. Like some *wts* genes of *E. stewartii*, *avrb6* was not required for bacterial growth *in planta*, but strongly affected watersoaking on its host.

The ion-channel defense model of the gene-for-gene hypothesis invokes a cell leakage factor as the product of an *avr* gene (Gabriel *et al.* 1988, Gabriel and Rolfe 1990). In this model, *avr* genes produce a protein or compound that opens an ion channel in the plant cell membrane that rapidly depolarizes the membrane, causing electrolyte leakage and host cell death. This gene-for-gene model is not inconsistent with the idea that the product of an *avr* gene might induce slower cell leakage on susceptible hosts. The only difference might be the allelic form of the host *R* gene. If the leakage were slow enough to avoid cascade amplification of a wound-response signal, changes in the osmotic gradient could cause a net loss of water from the cell and redistribution to the apoplast, thereby increasing the fluidity of the EPS (M. Essenberg and M. Pierce, personal communication). Increased fluidity or amounts of EPS may increase bacteria exuding onto the leaf surface through stomata (Thiers and Blank 1951). Another possible function is that the increased levels of necrosis induced by *avrb6* may serve to collapse the palisade layer and physically squeeze more bacteria onto the leaf surface.

CHAPTER 3

INACTIVATION OF MULTIPLE AVIRULENCE GENES IN *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM* CREATED A NONPATHOGENIC ENDOPHYTE OF COTTON

Introduction

Avirulence (*avr*) genes act as negative factors to confer avirulence and to limit host range of the pathogen on host plants carrying specific resistance (*R*) gene. As a result, most *avr* genes reported to date appear to be gratuitous and of no obvious selective value to the microbial plant pathogens (Gabriel *et al.* 1993). However, a few of them have demonstrated pleiotropic function(s). For example, *avrBs2* is required for full virulence of *Xanthomonas campestris* pv. *vesicatoria* on susceptible hosts (Kearney and Staskawicz 1990). More recently, *pthA* of *X. citri* and *avrb6* of *X. campestris* pv. *malvacearum* were also shown to have host-specific pathogenicity function in addition to avirulence function (Swarup *et al.* 1991, 1992; Chapter 2). Gene *pthA* is essential for *X. citri* strain 3213 to induce hyperplastic cankers on citrus specifically and confers that function to several other *X. campestris* pathovars. Similarly, *avrb6* is important for *X. campestris* pv. *malvacearum* strain XcmH to induce watersoaking on cotton specifically, and confers that function to other *X. campestris* pv. *malvacearum* strains. Both *pthA* and *avrb6* appear to contribute to the fitness of their respective pathogens on their hosts by

increasing release of the pathogens from plant leaves, thus affecting host range through increased dispersal.

Both *pthA* and *avrb6* are members of a large *Xanthomonas* avirulence (*avr*) and pathogenicity (*pth*) gene family, and both confer gene-for-gene avirulence to *X. campestris* pv. *malvacearum* on cotton (Swarup *et al.* 1993; De Feyter *et al.* 1993). Other members of the *avr/pth* gene family include *avrB4*, *avrb7*, *avrBln*, *avrB101* and *avrB102* of *X. campestris* pv. *malvacearum* (De Feyter and Gabriel 1991a, De Feyter *et al.* 1993), *avrBs3*, *avrBs3-2* and *avrBsP* of *X. campestris* pv. *vesicatoria* (Bonas *et al.* 1989, 1993; Canteros *et al.* 1991) and *avrXa5*, *avrXa7* and *avrXa10* of *X. oryzae* (Hopkins *et al.* 1992). Family members sequenced to date are 95%-98% identical to each other (Bonas *et al.* 1993; Hopkins *et al.* 1992; De Feyter *et al.* 1993). Most members of this gene family were isolated as avirulence genes with no evidence of pathogenicity function.

Xanthomonas campestris pv. *malvacearum* (Smith) Dye is the causal agent of bacterial blight of cotton, an economically important, world-wide disease. Six members of this gene family were previously isolated from pXcmH, a 90-kb plasmid of *X. campestris* pv. *malvacearum* strain XcmH. Southern hybridization analysis reveals at least five additional hybridizing fragments in XcmH besides the six plasmid-borne *avr* genes (De Feyter *et al.* 1993). Furthermore, all strains examined of *Xanthomonas campestris* pv. *malvacearum* that attack cotton carry at least 4-11 DNA fragments that hybridize with members of the *avr/pth* gene family, including many strains which carry no known *avr* genes (De Feyter *et al.* 1993). The purpose of this research was to clone

and to mutagenize all *avr* genes in *X. campestris* pv. *malvacearum* strain XcmH and to identify potential pleiotropic functions.

Materials and Methods

Bacterial strains, plasmids, and matings. The bacterial strains and plasmids used in this study are listed Table 3-1. Strains of *Escherichia coli* were grown in Luria-Bertani (LB) medium (Sambrook *et al.* 1989) at 37°C. Strains of *Xanthomonas* were grown in PYGM (peptone-yeast extract-glycerol-MOPS) medium at 30°C (De Feyter *et al.* 1990). For culture on solid media, agar was added at 15g/L. Antibiotics were used as previously described (refer to Chapter 2). To transfer plasmids from *E.coli* strains HB101 or DH5 α to *X. campestris* pv. *malvacearum*, helper plasmid pRK2073 and modifier plasmid pUFR054 were used as described (Feyter and Gabriel 1991a,b).

Recombinant DNA techniques. Total DNA isolation from *Xanthomonas* was as described (Gabriel and De Feyter 1992). Plasmids were isolated from *E. coli* by alkaline lysis methods (Sambrook *et al.* 1989). Restriction enzyme digestion, alkaline phosphatase treatment, DNA ligation and random priming reactions were performed as recommended by the manufacturers. Southern hybridization was performed by using nylon membranes as described (Lazo *et al.* 1987). Otherwise, standard recombinant DNA procedures were used (Sambrook *et al.* 1989).

Gene replacement and marker eviction. To carry out marker-exchange mutagenesis, a 3.8 kb *Bam*HI fragment containing a *nptI-sac* cartridge from pUM24

Table 3-1. Bacterial strains and plasmids used in this study

| Strain or Plasmid | Relevant Characteristics | Reference or Source |
|---|---|-------------------------------|
| <i>E. coli</i> | | |
| DH5 α | F ⁻ , <i>endA1</i> , <i>hsdR17</i> (r _k ⁻ m _k ⁺), <i>supE44</i> , <i>thi-1</i> , <i>recA1</i> , <i>gyrA</i> , <i>relA1</i> , ϕ 80 <i>dlacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169 | Gibco-BRL, Gaithersburg, MD |
| HB101 | <i>supE44</i> , <i>hsdS20</i> (r _k ⁻ m _k ⁺), <i>recA13</i> , <i>ara-14</i> , <i>proA2</i> , <i>lacY1</i> , <i>galK2</i> , <i>rpsL20</i> , <i>xyl-5</i> , <i>mtl-1</i> | Boyer & Roulland-Dussoix 1969 |
| ED8767 | <i>supE44</i> , <i>supF58</i> , <i>hsdS3</i> (r _k ⁻ m _k ⁺), <i>recA56</i> , <i>galK2</i> , <i>galT22</i> , <i>metB1</i> | Murray <i>et al.</i> 1977 |
| <i>X. campestris</i> pv. <i>malvacearum</i> | | |
| XcmH | Natural isolate from cotton from Oklahoma; carries six <i>avr</i> gene on pXcmH, and additional six potential <i>avr</i> genes. | De Feyter & Gabriel, 1991a |
| XcmH1005 | Spontaneous Rif derivative of XcmH | Chapter 2 |
| HM1.10 | <i>avrBln::nptI-sac</i> , marker exchange mutant of XcmH1005 | This study |
| HM1.15 | <i>avrb6::nptI-sac</i> , marker exchange mutant of XcmH1005 | This study |
| HM1.20 | <i>avrB5::nptI-sac</i> , <i>avrb6</i> ⁻ , marker exchange mutant of XcmH1005 | This study |
| HM1.20S | <i>avrB5</i> ⁻ , <i>avrb6</i> ⁻ marker-evicted mutant derived from HM1.20 | This study |
| HM1.26 | <i>Hc1::nptI-sac</i> , marker-exchange mutant of XcmH1005 | This study |
| HM1.32 | <i>Hc1</i> ⁻ , <i>avrBln</i> ⁻ , <i>avrB104</i> ⁻ marker exchange mutant of XcmH1005 | This study |
| HM1.34 | <i>avrBn::nptI-sac</i> , marker exchange mutant of XcmH1005 | This study |
| HM1.36 | <i>avrB103::nptI-sac</i> , marker exchange mutant of XcmH1005 | This study |
| HM1.38 | <i>avrB4</i> ⁻ , <i>avrb7</i> ⁻ , <i>avrBln</i> ⁻ , <i>avrB101</i> ⁻ , <i>avr102</i> ⁻ marker exchange mutant of XcmH1005 | This study |
| HM2.2 | <i>avrHc6</i> ⁻ , <i>avrB4</i> ⁻ , <i>avrb6</i> ⁻ , <i>avrb7</i> ⁻ , <i>avrBln</i> ⁻ , <i>avrB101</i> ⁻ , <i>avr102</i> ⁻ , marker exchange mutant of HM1.20S. | This study |
| HM2.2S | XcmH1005 (<i>avrHc6</i> ⁻ , <i>avrB4</i> ⁻ , <i>avrb6</i> ⁻ , <i>avrb7</i> ⁻ , <i>avrBln</i> ⁻ , <i>avrB101</i> ⁻ , <i>avr102</i> ⁻), marker-evicted mutant derived from HM2.2S. | This study |
| XcmN | Natural isolate from cotton from Upper Volta, Africa. | Gabriel <i>et al.</i> 1986 |
| Xcm1003 | Spc ^r Rif derivative of XcmN | De Feyter & Gabriel 1991a |

Table 3-1--continued.

| Strain or Plasmid | Relevant Characteristics | Reference or Source |
|-------------------|---|------------------------------|
| Plasmid | | |
| pRK2073 | pRK2013 derivative, npt::Tn7, Km ^r , Sp ^r , Tra ⁺ , helper plasmid | Leong <i>et al.</i> 1982 |
| pUFR004 | ColE1, Mob ⁺ , Cm ^r | De Feyter <i>et al.</i> 1990 |
| pUFR034 | IncW, Km ^r , Mob ⁺ , <i>lacZ</i> α ⁺ , Par ⁺ , <i>cos</i> | De Feyter <i>et al.</i> 1990 |
| pUFR042 | IncW, Km ^r , Gm ^r , Mob ⁺ , <i>lacZ</i> α ⁺ , Par ⁺ | De Feyter & Gabriel 1991a |
| pUFR047 | IncW, Gm ^r , Ap ^r , Mob ⁺ , <i>lacZ</i> α ⁺ , Par ⁺ | De Feyter <i>et al.</i> 1993 |
| pUFR054 | IncP, Tc ^r , Mob ⁺ , containing methylases <i>Xma</i> I and <i>Xma</i> III | De Feyter & Gabriel 1991b |
| pUFR103 | Cosmid clone carrying <i>avrB4</i> , <i>avrb6</i> . | De Feyter & Gabriel 1991a |
| pUFR106 | Cosmid clone carrying <i>avrB4</i> , <i>avrb6</i> , <i>avrB101</i> | De Feyter & Gabriel 1991a |
| pUFR107 | Cosmid clone carrying <i>avrb6</i> , <i>avrB101</i> , <i>avrBln</i> | De Feyter & Gabriel 1991a |
| pUFR109 | Cosmid clone carrying <i>avrB101</i> , <i>avrBln</i> | De Feyter & Gabriel 1991a |
| pUFR111 | Cosmid clone carrying <i>avrB101</i> , <i>avrBln</i> , <i>avrB102</i> | De Feyter & Gabriel 1991a |
| pUFR112 | Cosmid clone carrying <i>avrBln</i> , <i>avrB102</i> , <i>avrb7</i> | De Feyter & Gabriel 1991a |
| pUFR113 | Cosmid clone carrying <i>avrB102</i> , <i>avrb7</i> | De Feyter & Gabriel 1991a |
| pUFR115 | 7.5-kb fragment containing <i>avrB4</i> in pUFR042 | De Feyter & Gabriel 1991a |
| pUFR127 | 5-kb fragment containing <i>avrb6</i> in pUFR042 | De Feyter & Gabriel 1991a |
| pUFR142 | 9-kb fragment containing <i>avrB101</i> in pUFR047 | De Feyter <i>et al.</i> 1993 |
| pUFR156 | 12.9-kb fragment containing <i>avrBln</i> in pUFR042 | De Feyter <i>et al.</i> 1993 |
| pUFR157 | 11-kb fragment containing <i>avrB102</i> in pUFR042 | De Feyter <i>et al.</i> 1993 |
| pUFR163 | 10-kb fragment containing <i>avrb7</i> in pUFR042 | De Feyter <i>et al.</i> 1993 |
| pUFY1.48 | 8.4-kb fragment carrying <i>pthA::npt-sac</i> in pUFR047 | This study |
| pUFY10.1 | 8.1-kb fragment carrying <i>pthA::npt-sac</i> in pUFR004 | This study |
| pUFY31.46 | 8.5-kb fragment carrying <i>avrB103</i> in pUFR047 | This study |
| pUFY33.19 | 5-kb fragment carrying <i>avrB5</i> in pUFR047 | This study |
| pUFY36.8 | 8.6-kb fragment carrying Hc1 in pUFR047 | This study |
| pUFY36.26 | 5-kb fragment carrying <i>avrBn</i> in pUFR047 | This study |
| pUFY37.62 | 11-kb fragment carrying <i>avrB104</i> and <i>avrB5</i> on pUFR047 | This study |
| pUFY38.1 | 8-kb fragment carrying <i>avrB104</i> in pUFR047 | This study |
| pUM24 | <i>nptI-sac</i> marker in a 3.8-kb <i>Bam</i> HI fragment | Ried and Collmer 1987 |
| pXcm1.12 | Cosmid clone carrying Hc1 and <i>avrBn</i> | This study |
| pXcm1.21 | Cosmid clone carrying <i>avrB103</i> | This study |
| pXcm2.23 | Cosmid clone carrying <i>avrBn</i> | This study |
| pXcm2.12 | Cosmid clone carrying <i>avrB104</i> and <i>avrB5</i> | This study |
| pXcm2.11 | Cosmid clone carrying <i>avrB5</i> | This study |
| pXcm1.22 | Cosmid clone carrying Hc6 | This study |
| pZit45 | 4.5-kb fragment containing <i>pthA</i> in pUFR047 | Swarup <i>et al.</i> 1992 |

(Ried and Collmer 1987) was randomly ligated into a *BalI* site of *pthA* on pZit45. Recombinant plasmids were screened for an insertion in the middle of the tandemly repeated region, and pUFY1.48 was selected, in which the *nptI-sac* cartridge was found in the *BalI* site of repeat number 10 of *pthA*. An *SstI* fragment carrying the *pthA::nptI-sac* fusion from pUFY1.48 was recloned into the suicide vector pUFR004 (De Feyter *et al.* 1990), forming pUFY10.1. Marker-exchange mutants (*avr::nptI-sac*) were created by transferring pUFR10.1 to XcmH1005 and selecting for colonies resistant to kanamycin (15 μ g/ml) and sensitive to chloramphenicol (35 μ g/ml) and sucrose (5%). Some marker-exchange mutants were plated on the PYGM medium containing 5% sucrose and *nptI-sac* marker evicted strains were selected to allow further rounds of marker exchange mutagenesis.

Plant inoculations and bacterial growth *in planta*. Cotton (*Gossypium hirsutum* L.) lines used were Acala-44 (Ac44) and its congenic resistance lines AcB1, AcB2, AcB4, AcB5a, AcB5b, Acb6, Acb7, AcBIn and AcBIn3 as described (Swarup *et al.* 1992, De Feyter *et al.* 1993). Cotton plants were grown in the greenhouse, transferred to growth chambers before inoculation, and maintained under conditions as described (De Feyter and Gabriel 1991a). Bacterial suspensions of *X. campestris* pv. *malvacearum* (10^8 cfu/ml) in sterile tap water were gently pressure infiltrated into leaves of 4-6 week old cotton plants. Pathogenic symptoms were observed periodically 2-7 days after inoculation.

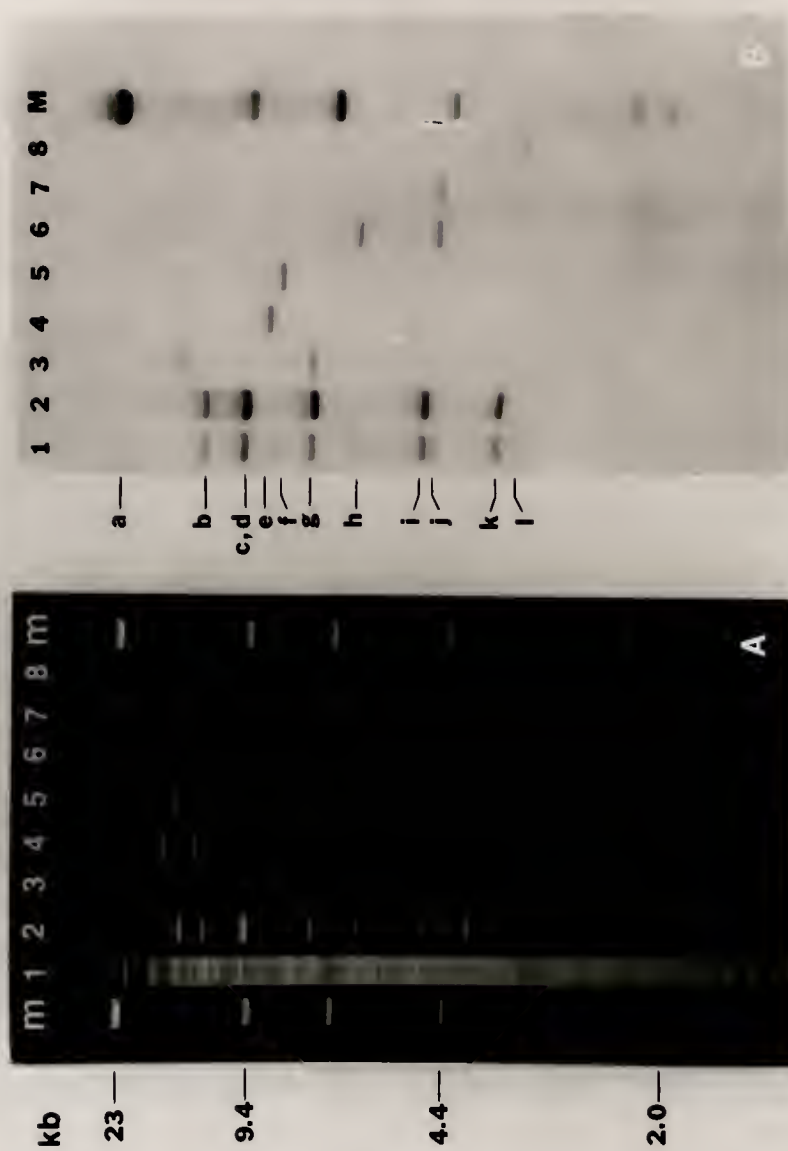
The growth of *X. campestris* pv. *malvacearum* in the susceptible cotton line Ac44 was determined as described (refer to Chapter 2). *In planta* growth experiments were

performed three times. Data points shown in Fig. 3-4 were the mean and standard error of three samples from one experiment. Bacterial populations released onto the leaf surface were quantified as described (refer to Chapter 2). These data are reported as the mean and standard error of three samples. Similar results were obtained in two independent experiments.

Results

Cloning of six potential *avr* genes from XcmH by hybridization. In addition to the six members of the *Xanthomonas avr/pth* gene family previously cloned from plasmid pXcmH of strain XcmH (De Feyter and Gabriel, 1991a), Southern blot analyses revealed the presence of six more DNA fragments in XcmH that hybridized to an internal *Bam*HI fragment from *avrb6* (Fig. 3-1). All of the fragments appeared to be large enough to carry functional members of the *avr/pth* gene family. Based on the intensity of hybridization and the fact that XcmH appears to carry only a single plasmid (pXcmH; De Feyter and Gabriel, 1991a), the additional hybridizing fragments appeared to be chromosomal. Colony hybridizations were carried out to isolate these chromosomal DNA fragments from a genomic library of XcmH (De Feyter and Gabriel 1991a) by probing with an internal *Bam*HI fragment from *avrb6*. Twenty-three out of seventy-two cosmid clones identified by colony hybridization were found to carry DNA fragments not found on pXcmH. These new, presumably chromosomal DNA fragments were readily distinguished from the DNA fragments that form pXcmH by restriction fragment length

Figure 3-1. Southern hybridization of cosmid DNAs containing six chromosomal *avr* genes after *Eco*RI and *Sst*I double digestion. The blot was probed with ³²-P labelled internal *Bam*HI fragment of *avrb6*. A, Ethidium bromide stained gel; B, autoradiograph resulting from Southern hybridization. Lane 1, XcmH genomic DNA; lane 2, pXcmH; lane 3, pXcm1.12 (Hcl1, upper band; and *avrBn*, lower band); lane 4, pXcm1.21 (*avrB103*); lane 5, pXcm2.23 (*avrBn*); lane 6, pXcm2.12 (*avrB104*, upper band; and *avrB5*, lower band); lane 7, pXcm2.11 (*avrB5*); lane 8, pXcm1.22 (Hc6); M, molecular marker. a, Hcl1; b, *avrB4*; c, *avrB101*; d, *avrB102*, e, *avrB103*; f, *avrBn*; g, *avrBln*; h, *avrB104*; i, *avrB7*; j, *avrB5*; k, *avrb6*; l, Hc6.



polymorphisms (Fig. 3-1). Six different chromosomal DNA fragments hybridizing with *avrb6* had been cloned and identified. These DNA fragments were arbitrarily named according to size as Hc1 - 6. Six cosmid clones were selected for further study: pXcm1.12 carried Hc1 and Hc3, pXcm1.21 carried Hc2, pXcm2.23 carried Hc3, pXcm2.12 carried Hc4 and Hc5, pXcm2.11 carried Hc5 only, and pXcm1.22 carried Hc6 (Fig. 3-1). Restriction digests of these clones revealed that Hc1, Hc2 and Hc3 were closely linked, and that Hc4 and Hc5 were closely linked.

Avirulence activity of hybridizing fragments. All cosmid clones were conjugally transferred to the widely virulent *X. campestris* pv. *malvacearum* strain Xcm1003 and tested on cotton cv. Acala 44 (Ac44) and 9 cotton lines, congenic with Ac44 and each carrying one of the following resistance genes: *B1*, *B2*, *B4*, *B5a*, *B5b*, *b6*, *b7*, *Bln* and *Bln3*. To localize members of the *avr/pth* gene family, all cosmid clones with avirulence activity were directionally subcloned into pUFR047 by *EcoRI* and *HindIII* digestion (these enzymes are not known cut within members of the family published to date). pXcm1.12 was subcloned to separate fragments Hc1 and Hc3, generating pUFY36.8 (Hc1) and pUFY36.26 (Hc3). Fragment Hc2 on pXcm1.21 was subcloned to form pUFY31.46. pXcm2.12 was subcloned to generate pUFY37.62, still carrying Hc4 and Hc5 on a single, 11 kb *EcoRI/HindIII* DNA fragment. Fragment Hc4 on pUFY37.62 was further subcloned to form pUFY38.1. Fragment Hc5 on pXcm2.11 was subcloned to form pUFY33.19. Since Hc6 on pUFY1.22 conferred no avirulence to Xcm1003, it was not evaluated further.

The indicated subclones were transferred to Xcm1003 and tested on the congenic cotton lines and on cotton line 20-3, carrying resistance gene *Bn*. The Hc1 and Hc6 DNA fragments conferred no detected phenotype to Xcm1003. The Hc2 and Hc4 DNA fragments conferred exactly the same avirulence specificity to Xcm1003 as did clones carrying *avrB101* (De Feyter *et al* 1993): avirulence on AcB5a and AcBIn3. Therefore we conclude that Hc2 and Hc4 carry two new members of the gene family, named *avrB103* and *avrB104*, respectively. The Hc3 DNA fragment on pXcm2.23 conferred avirulence to Xcm1003 on cotton lines containing resistance genes *Bn* or *BIn3*. Gene *avrBn* had previously been cloned from XcmH on pUFA-H1 and shown to confer avirulence on cotton lines containing *Bn* (Gabriel *et al*, 1986), but *avrBn* was not known to be a member of this *avr/pth* gene family. Restriction and Southern hybridization analyses (not shown) revealed that *avrBn* is a member of this gene family, and is carried on both pUFA-H1 and pXcm2.23. The Hc5 DNA fragment conferred avirulence to Xcm1003 on AcB5b and AcBIn3. Therefore we conclude that Hc5 carried a new member of the gene family, named *avrB5*.

Inactivation of *avr* genes in XcmH1005. To study the pleiotropic function of all members of the *avr/pth* gene family in XcmH1005 (a rifamycin mutant of XcmH), marker exchange-eviction mutagenesis was carried out to inactivate or delete each of the *avr* genes, singly or in combination. More than 140 mutants were generated and 60 of them were tested on 10 congenic cotton resistance lines and analyzed by Southern hybridization. Marker exchange-eviction mutants affecting all 10 *avr* gene were obtained. In Table 3-2 are listed selected mutants which exhibited altered phenotypes on

Table 3-2. Phenotypes of marker-exchange mutants of *Xanthomonas campestris* pv. *malvacearum* strain XcmH1005 on cotton.

| Xcm strains | Cotton cv. Acala-44 congenic lines | | | | | | |
|---|------------------------------------|------|-------|------|------|-------|--------|
| | Ac44 | AcB4 | AcB5a | AcB6 | AcB7 | AcBln | AcBln3 |
| XcmH1005 | + | + | - | - | - | - | - |
| HM1.10 (<i>avrBln::nptI-sac</i>) | + | + | - | - | - | + | + |
| HM1.15 (<i>avrB6::nptI-sac</i>) | + | - | - | + | - | - | - |
| HM1.20 (<i>avrB5⁻, avrB6⁻</i>) | + | - | ± | + | - | - | - |
| HM1.20S (<i>avrB5⁻, avrB6⁻</i>) | + | - | ± | + | - | - | - |
| HM1.38 (<i>avrB4⁻, avrB7⁻, avrBln⁻, avrB101⁻, avr102⁻</i>) | + | + | - | + | + | - | - |
| HM2.2 (<i>avrB4⁻, avrB5⁻, avrB6⁻, avrB7⁻, avrBln⁻, avrB101⁻, avr102⁻</i>) | 0/+ | 0/+ | 0/+ | 0/+ | 0/+ | 0/+ | - |
| HM2.2S (<i>avrB4⁻, avrB5⁻, avrB6⁻, avrB7⁻, avrBln⁻, avrB101⁻, avr102⁻</i>) | 0/+ | 0/+ | 0/+ | 0/+ | 0/+ | 0/+ | - |

Note: "+" strong watersoaking ; "+" weak watersoaking; "0/+" extremely weak watersoaking; "-" hypersensitive response (HR); "±" weak HR.

cotton. Southern analysis of 11 mutants was shown in Fig. 3-2. As predicted by gene-for-gene theory, mutations of *avrBln* (HM1.10), *avrb6* (HM1.15), or *avrB4* plus *avrb7* (HM1.38) in XcmH1005 resulted in the loss of *avr* specificity for cotton blight resistance genes *Bln*, *b6*, *B4*, and *b7*, respectively. As reported in the previous chapter, a single mutation of *avrb6* reduces the watersoaking ability of XcmH1005 on susceptible cotton lines. In contrast, mutation of *avrB4*, *avrb7* or *avrBln* did not affect watersoaking ability of XcmH1005 on Ac44. A mutation in *avrB5* (HM1.20) was shown to result in the same phenotypic defects as the mutation in *avrb6*, i. e., a loss of *avr* specificity for resistance gene *b6* and reduction of watersoaking on the susceptible cotton line Ac44. However, this mutation could not be complemented by the cloned *avrB5* gene, instead, was complemented by the cloned *avrb6*, indicating *avrb6* in HM1.20 was likely to be affected. Mutation of both *avrB5* and *avrb6* in HM1.20 and HM1.20S resulted a partial loss of avirulence specificity for cotton blight resistance gene *B5b*. Mutation of *avrB101*, *avrB102*, *avrB103* (HM1.36), *avrB104* (HM1.32), *avrBn* (HM1.34) or Hc1 (HM1.26) fragment resulted in no alteration of avirulence phenotype. This is consistent with the fact that the cotton resistance genes that react with these *avr* genes also react with other *avr* genes (gene-for-genes; De Feyter *et al.* 1993). No mutations affecting fragment Hc6 were recovered.

To inactivate a large number of *avr* genes in XcmH1005, the *nptI-sac* marker-evicted mutant HM1.20S (*avrB5*⁻, *avrb6*⁻) was subjected to a second round of marker exchange mutagenesis. The resulting mutant HM2.2 and marker evicted progeny HM2.2S had at least 7 *avr* genes (*avrB4*⁻, *avrB5*⁻, *avrb6*⁻, *avrb7*, *avrBln*⁻, *avrB101*⁻,

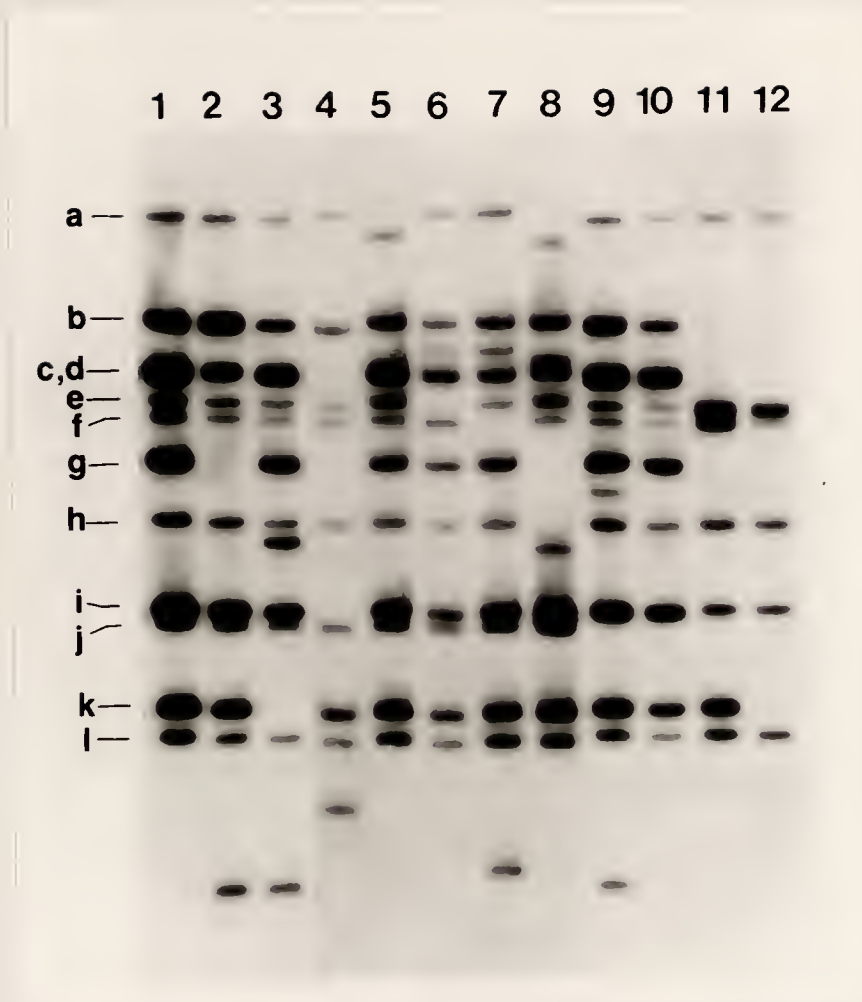


Figure 3-2. Southern hybridization of *Eco*RI and *Sst*I double digested genomic DNAs of XcmH mutants. The blot was probed with 32 P labelled internal *Bam*HI fragment of *avrb6*. Lane 1, XcmH; lane 2, HM1.10; lane 3, HM1.15; lane 4, HM1.38; lane 5, HM1.26; lane 6, HM1.36; lane 7, HM1.34; lane 8, HM1.32; lane 9, HM1.20; lane 10, HM1.20S; lane 11, HM2.2; lane 12, HM2.2S. a, Hc1; b, *avrB4*; c, *avrB101*; d, *avrB102*, e, *avrB103*; f, *avrBn*; g, *avrBln*; h, *avrB104*; i, *avrb7*; j, *avrB5*; k, *avrb6*; l, Hc6.

avr102) inactivated. These two mutants not only lost *avr* specificity on most of cotton resistance lines, but also lost nearly all watersoaking ability on the susceptible line Ac44 (Tab. 3-2, Fig. 3-3).

Despite repeated attempts to inactivate all members of the gene family in XcmH, we were unable to reduce the number of members below five. In all cases, members of the family appeared to rearrange to form new members, as evidenced by the appearance of new DNA fragments of sufficient size to encode a member, and often accompanied by the appearance of a new avirulence specificity phenotype (data not shown). Also in all cases, at least one member remained plasmid-borne, as evidenced by comparatively strong hybridization intensities characteristic of the plasmid-borne genes.

Effect of loss of watersoaking on bacterial growth *in planta*. Since *avrb6* mutants of XcmH1005 that had lost significant watersoaking ability on Ac44 were shown to be unaffected in growth *in planta* (refer to Chapter 2), we evaluated growth of HM2.2S on Ac44 in comparison with the parental strain XcmH1005. While inactivation of 7 *avr* genes resulted in a loss of nearly all watersoaking ability on cotton, *in planta* growth rate and yield of HM2.2S was not affected (Fig. 3-4). However, the amount of HM2.2S released onto the leaf surface was 1600 times less than that of XcmH1005. Only 0.007% ($8.8 \pm 3.0 \times 10^4$ cfu/cm² out of $1.18 \pm 0.24 \times 10^9$ cfu/cm²) of HM2.2S bacteria present in the infected leaf tissue were released onto the leaf surface, whereas 9.2% ($1.42 \pm 0.15 \times 10^8$ cfu/cm² out of $1.55 \pm 0.23 \times 10^9$ cfu/cm²) of the XcmH1005 bacteria were released onto the surface. Therefore, the mutant HM2.2S was basically a nonpathogenic endophyte of cotton.

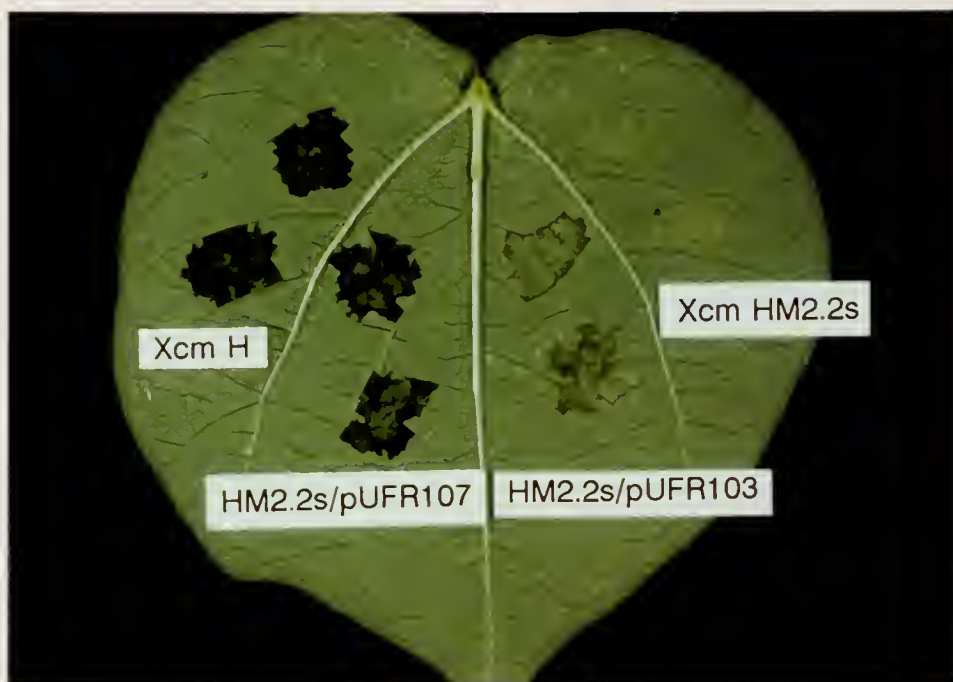


Figure 3-3. Effect of *avr* genes on watersoaking symptoms caused by *X. campestris* pv. *malvacearum* on cotton susceptible line Ac44.

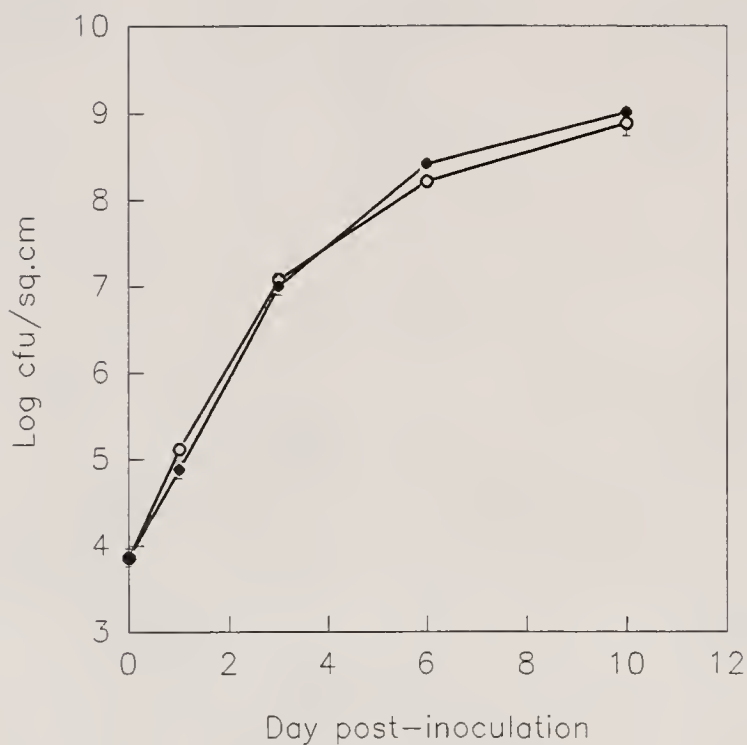


Figure 3-4. Inactivation of multiple *avr* genes in *X. campestris* pv. *malvacearum* did not affect bacterial growth *in planta* on cotton susceptible line Ac44. Filled circle, XcmH1005; open circle, HM2.2S.

Additive effect of different *avr* genes on watersoaking ability of XcmH1005.

Complementation tests were carried out to further analyze the potential pleiotropic pathogenicity function of 12 *avr* genes isolated from XcmH. As shown in Fig. 3-5, when plasmids carrying single *avr* genes isolated from XcmH were introduced into the mutant HM2.2S, only *avrb6* was able to partially complement the watersoaking defect of HM2.2S. None of the other 9 *avr* genes or Hc1 or Hc6 fragments were able to even partially complement the watersoaking defect in HM2.2S when introduced individually. However, when plasmids carrying two or three of these same *avr* genes (not necessarily including *avrb6*) were introduced into HM2.2S, many were able to partially complement the watersoaking defect (Figs. 3-3 and 3-5). For example, plasmids containing *avrBln* or *avrB101* alone conferred no watersoaking activity, but plasmids containing both *avrBln* and *avrB101* together exhibited watersoaking activity. Cosmid clones (pUFR106 and pUFR107) carrying *avrb6* and at least two other *avr* genes were able to fully complement the watersoaking defects of HM2.2S (Fig. 3-3). Therefore, at least three of the pXcmH *avr/pth* genes were redundant in terms of pathogenicity function.

To exclude the possibility that some watersoaking genes were linked to *avr* genes on these complementing cosmid clones, subcloning of pUFR107 was carried out. Activity assays of regions between the *avr* genes did not reveal any watersoaking activity when introduced into HM2.2S.

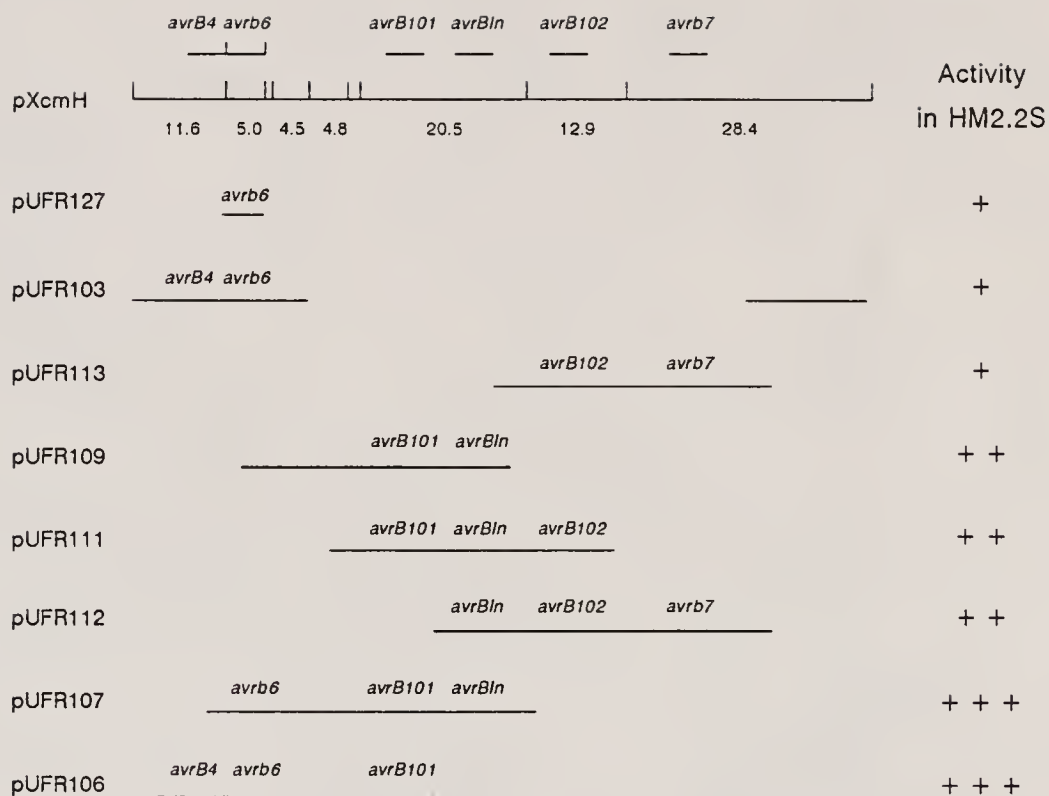


Figure 3-5. Pleiotropic watersoaking function of cloned XcmH *avr* genes in mutant HM2.2S. * HM2.2S was nearly asymptomatic on susceptible cotton line Ac44; + weak watersoaking activity; ++ intermediate level watersoaking activity; +++ full watersoaking activity (as wild type XcmH).

Discussion

Nearly all of the *avr* genes reported from the genus *Xanthomonas* are members of a single, highly homologous gene family, and with the exception of *pthA* (pathogenicity) of *X. citri* (Swarup *et al.* 1991, 1992), all of the other published members were identified and cloned as *avr* genes. Southern analyses have shown that members of this *avr/pth* gene family exist in multiple copies (from 4 to 11) in all tested *X. campestris* pv. *malvacearum* strains isolated from cotton, but that some strains, particularly those from Africa, appear to lack functional avirulence (De Feyter *et al.* 1993). For example, the African strain Xcm1003 used in this study contains at least five hybridizing fragments, but is virulent on all tested cotton resistance lines. Of course it is possible that the hybridizing fragments in a single strain are all nonfunctional, but it is more difficult to explain the presence of from 4 - 11 hybridizing DNA fragments present in all known *X. campestris* pv. *malvacearum* strains if they encoded no function beneficial to the pathogen.

All 12 DNA fragments that hybridize with an internal fragment from the gene family have now been isolated from a single *X. campestris* pv. *malvacearum* strain, XcmH. Six of them are clustered on a 90-kb indigenous plasmid, pXcmH, and were originally isolated as functional *avr* genes, although two of them (*avrb6* and *avrb7*) were noted to confer pleiotropic pathogenicity (watersoaking) function (De Feyter and Gabriel 1991a; De Feyter *et al.* 1993). We report here the cloning of the other six hybridizing fragments that appear to be from the chromosome. One of these fragments encodes the

previously described *avrBn* (Gabriel *et al* 1986). Three of these encode previously undescribed *avr* genes (*avrB5*, *avrB103* and *avrB104*), and the remaining two fragments conferred no avirulence on cotton and were presumably non-functional.

Mutations of all ten *avr* genes in XcmH1005 were achieved by marker exchange-eviction mutagenesis. With the exception of *avrb6*, mutations of individual members of the *avr/pth* gene family in XcmH1005 had no obvious effect on watersoaking on susceptible cotton lines. Mutation of *avrb6* resulted in a significant loss of watersoaking ability of XcmH1005. Although *avrb6* appeared to be the major pathogenicity gene among 10 homologous *avr* genes in the strain XcmH, many XcmH1005 *avr* genes also contributed to watersoaking function of XcmH1005 on cotton. Therefore these *avr* genes also function pleiotropically as *pth* genes. Mutant HM2.2S had at least seven *avr* genes (including *avrb6*) inactivated, and completely lost the ability to induce watersoaking on mature cotton leaves. Since HM2.2S suffered deletions of *avr* genes and adjacent DNA fragments, mutational analysis alone did not rule out the potential involvement of closely-linked virulence genes. However, complementation tests confirmed that *avr/pth* family members fully complemented the watersoaking defect(s) of HM2.2S. Individually, none of the 10 *avr/pth* genes, other than *avrb6*, could even partially complement the nonpathogenic mutant strain HM2.2S in visual assays. However, DNA fragments carrying multiple *avr/pth* genes from plasmid pXcmH were able to partially complement the pathogenic defect of HM2.2S, even if they did not carry *avrb6*, demonstrating that other *avr/pth* genes from XcmH also contributed to its pathogenicity.

Some *avr/pth* genes appeared to have stronger pathogenicity function than others in HM2.2S, and all six pXcmH genes appeared able to contribute to pathogenicity on cotton, when tested in combinations. However, no individual *avr/pth* gene, except *avrb6*, exhibited *pth* function in HM2.2S. Full restoration of the watersoaking defect of HM2.2S required *avrb6* plus two other pXcmH *avr/pth* genes. Thus, the cotton pathogen XcmH contained one major and multiple minor genes from the same *avr/pth* gene family. Some of the minor *avr/pth* genes appeared to be redundant in terms of watersoaking function. Therefore mutation of one or few of minor *avr/pth* genes would not affect the watersoaking and release of the pathogen, and presumably, the fitness of the pathogen.

In contrast to *avrBs2* of *X. campestris* pv. *vesicatoria* (Kearney and Staskawicz 1990), there was no evidence that the *avr/pth* genes of XcmH are even slightly involved in bacterial growth *in planta*. Instead, they were involved in the induction of watersoaking symptoms and associated release of 9.2% of the total bacteria of the leaf onto the leaf surface, presumably aiding in dispersal. Mutant HM2.2S, with at least seven *avr/pth* genes destroyed, released 1,600 times less bacteria to the leaf surface than the wild type. Although HM2.2S was asymptomatic on cotton, it maintained the same level of bacterial growth *in planta* as the wild type strain. Therefore, inactivation of multiple *avr/pth* genes in *X. campestris* pv. *malvacearum* created a nonpathogenic endophyte of cotton. In the case of *X. campestris* pv. *malvacearum*, pathogenicity and host tissue destruction appears to be beneficial for the reproductive fitness of the pathogen. Since *X. campestris* pv. *malvacearum* spreads primarily by rain splash, it seems doubtful that an endophyte could disseminate efficiently in natural field situations.

The lack of correlation between disease symptoms and growth *in planta* has been observed with other plant pathogens. Indole acetic acid-deficient strains of *Pseudomonas syringae* pv. *savastanoi* failed to elicit gall on oleander but exhibited similar growth patterns as the gall-eliciting wild-type strain (Smidt and Kosuge 1978). The *lemA* gene, which encodes a two-component regulator, is required by *P. syringae* pv. *syringae* for disease lesion formation on bean plants, but not bacterial growth within or on the leave of bean (Hrabak and Willis 1994; Willis *et al.* 1990). Mutation of the fungal pathogen *Colletotrichum magna*, resulted in a strain which is not able to induce pathogenic symptoms, but grows in host tissue as an endophyte and retains the wild-type host range (Freeman and Rodriguez 1993).

A growing number of *Xanthomonas* virulence / pathogenicity genes are not *hrp* (hypersensitive response and pathogenicity) genes, but instead are host-specific determinants of disease and/or host range (Gabriel *et al.*, 1993). For example, several genes affecting host range were identified and complementing DNA clones isolated from *X. campestris* pv. *citrumelo* (Kingsley *et al.*, 1993) and *X. campestris* pv. *translucens* (Waney *et al.*, 1992). These genes are host specific and affect growth in some hosts, but not in other hosts. Other non-*hrp* *Xanthomonas* pathogenicity genes, such as *pthA* of *X. citri* and *avrb6* of *X. campestris* pv. *malvacearum* are host specific and strongly affect elicitation of pathogenic symptoms. From the present study, it appears that many members of the *avr/pth* gene family of *X. campestris* pv. *malvacearum* are host-specific determinants of disease and host range on cotton. Genetically, *pthA* of *X. citri* and the *avr/pth* genes of *X. campestris* pv. *malvacearum* resemble some *Rhizobium* host-specific

nodulation genes which are required for host range on some hosts, but which can also confer avirulence when transferred to other *Rhizobium* strains with a different host range (Debelle *et al.* 1988; Faucher *et al.* 1989; Lewis-Henderson and Djordjevic 1991).

The pleiotropic function and apparent fitness value of some *avr* genes do not imply that most *avr* genes have selective value for a specific plant pathogenic strain. In fact, most *avr* genes reported to date appear to be dispensible and have no obvious selective value to the pathogen (Gabriel *et al.* 1993). At least some members of the *Xanthomonas avr/pth* gene family are found on self-mobilizing plasmids (for example, Bonas *et al.* 1989), and their presence in some strains of a pathovar may be due to a coincidental linkage with another (selected) factor, such as copper resistance, on the same plasmid. Once horizontally transferred to a different strain, these genes may not remain stable enough to retain *pth* function specific for some ancestral host. In both *X. campestris* pv. *malvacearum* and *X. citri*, members of the *avr/pth* gene family mutate at very high frequency (approximately 10^{-4}) through intergenic or intragenic recombination (De Feyter *et al.* 1993; Chapter 4). High frequency recombination provides a powerful mechanism to generate variation and horizontal gene transfer provides a powerful mechanism to disseminate that variation in the genus. It seems likely that many *avr* genes carried by bacterial plant pathogens may result from variation in redundant and/or unnecessary virulence/pathogenicity genes which are selectively neutral in natural populations.

CHAPTER 4

INTRAGENIC RECOMBINATION OF A SINGLE PLANT PATHOGEN GENE PROVIDES A MECHANISM FOR THE EVOLUTION OF NEW HOST SPECIFICITIES

Introduction

In gene-for-gene interactions between plants and microbial plant pathogens, host plant resistance results from the genetic recognition of resistance (*R*) genes in the plant and avirulence (*avr*) genes in the pathogen (Keen 1990; Gabriel and Rolfe 1990). It is unknown why microbial plant pathogens carry genes that function to limit virulence, since the majority of these genes are dispensable (Gabriel *et al.* 1993). For example, to avoid plant resistance, plant pathogens evade *R* gene recognition by deletion (De Feyter *et al.* 1993), mutation (Kobayashi *et al.* 1990; Joosten *et al.* 1994) or transposon insertion (Kearney *et al.* 1988) of *avr* genes. These reports all involve natural mechanisms whereby avirulence may be lost. Nevertheless, mutational analyses reveal that gains in avirulence, including new avirulence specificities, are generated in at least some pathogens at approximately the same frequency as they are lost (Statler 1985). Although presumably an *avr* gene may be reactivated by reverse mutation, there are no reports of natural mechanisms whereby new avirulence specificities or new *avr* genes are generated. The mechanisms by which new *avr* genes evolve are unknown.

Xanthomonas citri is the causal agent of citrus canker disease, which occurs worldwide and is subject to eradication and quarantine regulations in many countries. A pathogenicity gene, *pthA*, is essential for the pathogen to cause hyperplastic canker symptoms on citrus (Swarup *et al.* 1991). Furthermore, when transferred to other xanthomonads, *pthA* confers ability to induce hyperplastic cankers on citrus and a hypersensitive response (HR) on other hosts (Swarup *et al.* 1992). Therefore, *pthA* exhibits pleiotropic pathogenicity and avirulence functions. Southern hybridization, restriction analysis and partial DNA sequencing has shown that *pthA* belongs to a major *avr* gene family widespread in the genus *Xanthomonas* (Swarup *et al.* 1992). Members of this gene family include *avrBs3*, *avrBs3-2*, and *avrBsP* (a truncated form of *avrBs3-2*) of *X. c.* pv. *vesicatoria* (Bonas *et al.* 1989, 1993; Canteros *et al.* 1991), *avrB4*, *avrb6*, *avrb7*, *avrBIn*, *avrB101* and *avrB102* of *X. c.* pv. *malvacearum* (De Feyter and Gabriel 1991; De Feyter *et al.* 1993), and *avrXa10* and *avrXa7* of *X. oryzae* pv. *oryzae* (Hopkins *et al.* 1992). Among these, only *avrb6* and *pthA* are known to have pleiotropic pathogenicity functions (Swarup *et al.* 1992; De Feyter and Gabriel 1991; also refer to Chapter 2). The most striking feature of this gene family is the nearly identical, 102bp tandem repeats in the central portion of the genes. Deletion analyses have shown that the avirulence specificity of *avrBs3* is determined by the 102bp repetitive motifs of that gene (Herbers *et al.* 1992). By swapping the internal repeated regions between *pthA* and *avrb6*, the 102 bp tandem repeats are found to determine both avirulence and pathogenic specificities (refer to Chapter 2).

In most organisms, repetitive regions of DNA are known to be active sites for homologous recombination (Albertini *et al.* 1982; Petes and Hill 1988). The high frequency of such events causes them to be major genome modifying forces in evolution. Intergenic recombination among members of this *avr/pth* gene family was proposed to explain the high frequency of race change mutation found in *X. c. pv. malvacearum* (De Feyter *et al.* 1993). Since both pathogenic and avirulence specificities are determined by the 102bp tandem repeats, it seemed reasonable to suppose that homologous recombination between genes and among repeats of the same gene might lead to an acceleration in the evolution of this *Xanthomonas avr/pth* gene family, resulting in new host specificities for the pathogen. In this study, we analyzed the nucleotide sequence of *pthA* and demonstrate that intragenic recombination of a single gene can provide a genetic mechanism for the creation of new *avr/pth* genes.

Materials and Methods

Bacterial strains, plasmids and matings. *Escherichia coli* strains DH5 α (Gibco-BRL, Gaithersburg, MD), JM83 (Yanisch-Perron *et al.* 1985) and derivatives were grown in Luria-Bertani (LB) medium (Sambrook *et al.* 1989) at 37°C. *Xanthomonas citri* strain 3213 (causes hyperplastic cankers on citrus; Swarup *et al.* 1991, 1992), *X. campestris* pv. *citrumelo* strain 3048 (causes water-soaked leaf spots on citrus and common bean; Swarup *et al.* 1991, 1992), *X. campestris* pv. *malvacearum* strain Xcm1003 (causes water-soaked leaf spots on cotton; De Feyter and Gabriel 1991) and

derivatives were grown in PYGM (peptone-yeast extract-glycerol-MOPS) medium at 30°C (De Feyter *et al.* 1990). Antibiotics were used as described in previous chapters. Triparental matings were carried out to transfer plasmids from *E.coli* DH5 α to various *Xanthomonas* strains by using pRK2013 or pRK2073 as helper plasmids as described (Swarup *et al.* 1991; De Feyter and Gabriel 1991a). To transfer plasmids into Xcm1003, the modifier plasmid pUFR054 carrying *XcmI* and *XcmIII* methylase genes was used to increase the transfer frequency (De Feyter and Gabriel 1991b).

DNA sequence analysis. The 4.1 kb *SalI* fragment carrying *pthA* in pZit45 (Swarup *et al.* 1992) was recloned into the pGEM 7Zf(+) vector (Promega, Madison, WI) to yield pUFY14.5. Sets of overlapping, unidirectional deletion subclones were generated in pUFY14.5 using exonuclease III and mung bean nuclease as described by Promega (Madison, WI). DNA sequencing was performed by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville. Some sequencing was determined by using the dideoxy nucleotide chain termination technique with the Amersham (Arlington Heights, IL) multiwell microtiter plate DNA sequencing system RPN1590. The sequence was analyzed using the GCG package (version 7.00) by Genetics Computer Group, Inc., Madison, WI.

Gene replacement and selection of marker-evicted strains. Gene *pthA* on pZit45 was mutated by ligating a 3.9 kb *BamHI* fragment containing a *npI-sac* cartridge (Ried and Collmer 1987) into a *BalI* site in one of the 102bp tandem repeats of *pthA*. One of the resulting transcriptional fusions, pUFY1.48, carried the *npI-sac* cartridge inserted into repeat #10 of *pthA* and was selected for further study. An *SstI* fragment carrying

the *pthA::nptI-sac* fusion from pUFY1.48 was recloned into "suicide" vector pUFR004 (*colE1*, *mobP*⁺, *cat*⁺; De Feyter *et al.* 1990), forming pUFY10.1. Marker-exchange mutant Xc1.2 (*pthA::nptI-sac*) was created by transferring pUFR10.1 to *X. citri* 3213 and selecting for colonies resistant to kanamycin (15 µg/ml) and sensitive to chloramphenicol (35 µg/ml) and sucrose (5%). Marker-evicted strains of *X. citri* were selected by plating Xc1.2 on PYGM medium containing 5% sucrose and selecting colonies resistant to sucrose. Colonies were then screened for sensitivity to kanamycin. Recombinant, marker-evicted derivatives of pUFY1.48 were selected in *X. campestris* pv. citrumelo 3048, *E. coli* DH5α (*recA*⁻) and *E. coli* JM83 (*recA*⁺) by plating on PYGM or LB medium containing 5% sucrose. Sucrose-resistant colonies were screened for sensitivity to kanamycin.

To calculate recombination frequencies, XcM1.2, 3048/pUFY1.48, DH5α/pUFY1.48 and JM83/pUFY1.48 were grown in liquid media in the presence of appropriate antibiotics to densities of 10⁹ cells/ml. Serial dilutions were then plated on media with and without 5% sucrose. Recombination frequencies were calculated by dividing the number of sucrose-resistant (also kanamycin-sensitive) colonies by the number of colonies grown on the medium without sucrose selection (Xu *et al.* 1988). Data shown in the results were the mean and standard error of four replicates obtained from two independent experiments.

Plant inoculations. Citrus (*Citrus paradisi* 'Duncan', grapefruit), common bean (*Phaseolus vulgaris* 'California Light Red') and cotton (*Gossypium hirsutum* L.) plants were grown under greenhouse conditions. Cotton lines used were Acala-44 (Ac44) and

its congeneric resistance lines AcB1, AcB2, AcB4, AcB5, Acb6, Acb7, AcBIn and AcBIn3 as described (De Feyter *et al.* 1993, Swarup *et al.* 1992). Plant inoculations involving *X. citri* or *pthA* or derivatives of *pthA* were carried out in BL-3P level containment (refer to Federal Register Vol.52, No.154, 1987) at the Division of Plant Industry, Florida Department of Agriculture, Gainesville. Bacterial suspensions were standardized in sterile tap water to 10^8 cfu/ml and pressure infiltrated into the abaxial leaf surface of the plants. Plant inoculations were repeated at least three times on citrus, and two times on bean and cotton.

Results

Sequence analysis of *pthA*. The complete DNA sequence of *pthA* was determined and shown in Fig. 4-1. DNA sequence comparisons revealed that *pthA* of *X. citri* is 98% identical to *avrBs3* and *avrBs3-2* of *X. campestris* pv. *vesicatoria* (Bonas *et al.* 1989, 1993), 97% identical to *avrb6* of *X. campestris* pv. *malvacearum* (De Feyter *et al.* 1993), and 95% identical to *avrXa10* of *X. oryzae* (Hopkins *et al.* 1992) along its entire length. As observed with all previously sequenced members of the gene family (Bonas *et al.* 1993; De Feyter *et al.* 1993), *pthA* is flanked by nearly identical 62bp terminal inverted repeats that precisely define the limits of homology. The predicted amino acid sequence encoded by *pthA* is presented in Fig. 4-2. PthA has a calculated molecular weight of 122kD and an isoelectric point of 7.72. The primary region which differentiates the predicted PthA sequence from the predicted sequences of other members of the family

Figure 4-1. The complete DNA sequence of *pilA*. Indicated in the figure are the left and right 62-bp terminal inverted repeats (IRL and IRR) flanking *pilA* gene, -35 and -10 elements, Shine-Dalgarno (SD) ribosome-binding site, methionine (M) start codon, the first 102-bp tandem repeat, and stop codon (*).

[illegible]

Figure 4-2. Predicted amino acid sequence of PthA. The 17.5 internal tandem repeats are aligned and numbered for comparison. Letters A through K represent different types of repeats. * indicates the repeats unique for PthA protein. Bold and underlined letters indicate an amino acid deletion, substitution or specific combinations of amino acid residues within a given repeat that is unique to PthA (and the combination is not found in any repeat in any previously published member of the gene family). Three nuclear localization consensus sequences are double underlined.

1 MDPIRSRTPS PARELLPGPQ PDGVQPTADR GVSPAGGPL DGLPARRTMS RTRLPSPPAP
 61 SPAFSAGSFS DLLRQFDPSL FNTSLFDSL PFGAHTTEAA TGEWDEVQSG LRAADAPPPT
 121 MRVAVTAARP PRAKPAPRRR AAQPSDASPA AQVDLRTLGY SQQQQEKIKP KVRSTVAQHH
 181 EALVGHGFTH AHIVALSQHP AALGTAVKY QDMIAALPEA THEAIVGVGK QWSGARALEA
 241 LLTVAGELRG PPLQLDTGQL LKIAKRGVTV AVEAVHAWRN ALTGAPLN

| | | |
|---|----|-------|
| LTPEQVVAIA SNIGGKQALE TVQRLLPVLC QAHG | 1 | (A) |
| LTPEQVVAIA SN_GGKQALE TVQRLLPVLC QAHG | 2 | (B) * |
| LTPEQVVAIA SNIGGKQALE TVQRLLPVLC QAHG | 3 | (A) |
| LTPEQVVAIA SNIGGKQALE TVQRLLPVLC QAHG | 4 | (A) |
| LTPEQVVAIA SNIGGKQALE TVQALLPVLC QAHG | 5 | (C) |
| LTPEQVVAIA SNGGGKQALE TVQRLLPVLC QAHG | 6 | (D) |
| LT <u>P</u> DQVVAIA SH <u>D</u> GGKQALE TVQRLLPVLC <u>Q</u> AHG | 7 | (E) * |
| LT <u>P</u> QVVAIA SNGGGKQALE TVQRLLPVLC QAHG | 8 | (F) |
| LTPEQVVAIA SHDGGKQALE TVQRLLPVLC QAHG | 9 | (G) |
| LTPEQVVAIA SNGGGKQALE TVQRLLPVLC QAHG | 10 | (D) |
| LTPEQVVAIA SNGGGKQALE TVQRLLPVLC QAHG | 11 | (D) |
| LTPEQVVAIA SNGGGKQALE TVQRLLPVLC QAHG | 12 | (D) |
| LT <u>L</u> DQVVAIA S <u>N</u> GGGKQALE TVQRLLPVLC QAHG | 13 | (H) * |
| LTPEQVVAIA SNSGGKQALE TVQRLLPVLC QAHG | 14 | (I) |
| LT <u>P</u> <u>D</u> QVVAIA SH <u>D</u> GGKQALE TVQRLLPVLC <u>Q</u> AHG | 15 | (E) * |
| LTPEQVVAIA SHDGGKQALE TVQRLLPVLC QAHG | 16 | (G) |
| LTPEQVVAIA <u>C</u> NGGGKQALE TVQRLLPVLC QAHG | 17 | (J) * |
| LTPEQVVAIA SNGGGRPALE SIVAQLSRPD PALA | 18 | (K) |

900 A LTNDHLVALA CLGGRPALDA VKKGLPHAPA LIKRTNRRIP ERTSHRVADH
 951 AQVVRVLGFF QCDSHPAQAF DDAMTQFGMS RHGLLQLFRR VGVTELEARS GTLPPASQRW
 1011 DRILQASGMK RAKPSPTSTQ TPDQASLHAF ADSLERDLDA PSPTHEGDQR RASSRKRSRS
 1071 DRAVTGPSAQ QSFEVRAPEQ RDALHLPLSW RVKRPRTSIG GGLPDPGTPT AADLAASSTV
 1131 MREQDEDPFA GAADDFFAFN EEELAWLMEL LPQ*

is in the central portion of the protein, characterized by 17.5 nearly identical, tandemly arranged, 34 amino acid repeats. As shown in Fig. 4-2, these repeats may be classified into 11 types (A-K) depending on slight differences in sequence. Among them, repeat types B, E, H, J are only found in PthA, and not in the predicted peptide sequences of any other published family member. Repeat 2 (type B) resulted from an amino acid deletion and repeat 17 (type J) resulted from an amino acid substitution; neither this deletion nor this substitution has been observed in any other member of the gene family. Repeat types E (repeats 7 and 15) and H (repeat 13) resulted from unique combinations of amino acids in specific positions. In addition to the presence of unique repeats, PthA differs from its homologues in the arrangement of repeat types within the region.

Gene replacement and selection of *pthA* recombinants in *X. citri*. *X. citri* mutant Xc1.2 (*pthA::nptI-sac*) was generated from wild type strain 3213 by marker exchange, using pUFY10.1. Marker exchange was confirmed by Southern hybridization analyses (Fig. 4-3, compare lanes 1 and 2). When inoculated on plants, Xc1.2 was unable to induce cankers on citrus or to induce an HR on bean (Table 4-1). By plating Xc1.2 on a sucrose-containing medium and screening for sucrose resistant and kanamycin sensitive colonies, many marker-evicted strains of *X. citri* were obtained at a frequency of $5.22 \pm 1.8 \times 10^{-5}$.

To examine the recombination events, total DNAs were extracted from twenty-four marker-evicted derivatives of Xc1.2, digested with *Bam*HI and probed with the ³⁵P-labelled internal *Stu*I/*Hinc*II fragment (containing the tandemly repeated region) of *pthA*.

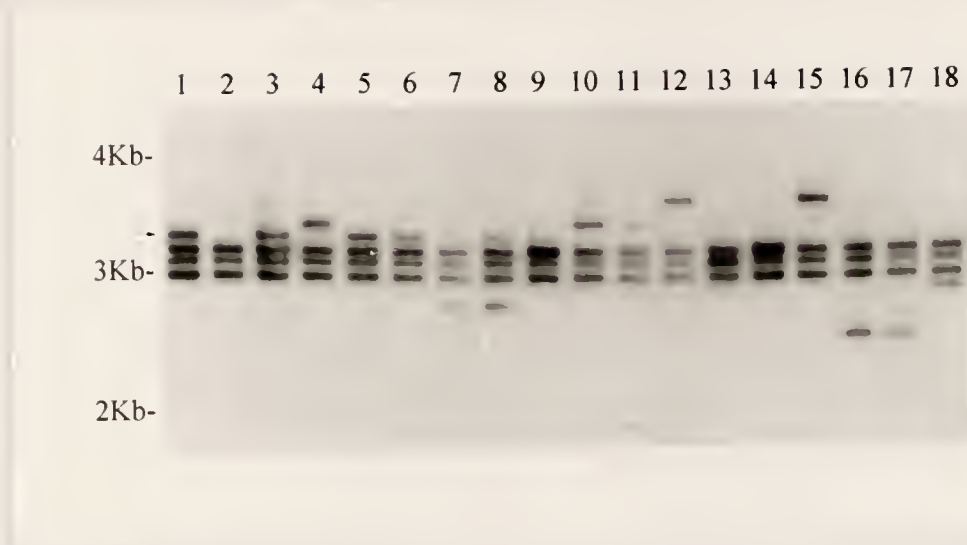


Figure 4-3. Southern hybridization of *Bam*HI digested, total DNA from *X. citri* strain 3213 and its *pthA* mutant derivatives. The blot was probed with the internal 2kb *Stu*I/*Hinc*II fragment (containing the tandemly repeated region) of *pthA*. Strain 3213 carries four *Bam*HI fragments which hybridize to *pthA*; the largest fragment, indicated by the arrow, encodes *pthA*. Not shown in lane 2 are two additional hybridizing fragments (1.8 and 1.6 kb in size), resulting from the additional *Bam*HI site in *nptI-sac*. Lane 1, 3213; lane 2, Xc1.2 (*pthA::nptI-sac*); lanes 3-18, *nptI-sac*-evicted derivatives of Xc1.2: XcS1, XcS15, XcS19, XcS24, XcS27, XcS30, XcS12, XcS13, XcS17, XcS18, XcS26, XcS29, XcS32, XcS23, XcS34, XcS26 and XcS21, respectively.

Table 4-1. Phenotypes of *Xanthomonas citri* marker-evicted strains on citrus and bean.

| Strains | Host | |
|---------------------------|--------|------|
| | citrus | bean |
| Xc3213 | C | HR |
| Xc1.2 | 0 | 0 |
| XcS1, XcS9, XcS14 & XcS15 | C | HR |
| XcS5 | C | 0 |
| XcS17 & XcS20 | wC | wHR |
| XcS12, XcS13 & XcS24 | HR | wHR |
| XcS7 | wHR | wHR |
| XcS6 & XcS11 | wHR | 0 |
| XcS2* | 0 | 0 |

*: Additional 11 strains (XcS3, XcS4, XcS8, XcS10, XcS16, XcS18, XcS19, XcS20, XcS21, XcS22 and XcS23); C, canker lesions; wC, weak canker; HR, hypersensitive reaction; wHR, weak hypersensitive reaction; 0, no symptoms.

As shown in Fig. 4-3, the different derivatives of Xc1.2 exhibited different sized bands (including those similar in size to *pthA*), indicating that new *pthA* homologues were regenerated by homologous recombination among the tandem repeats of *pthA*. There was no evidence of recombination between *pthA* and the other three DNA fragments that hybridize with *pthA*, but the presence of the other three fragments raised the possibility of intergenic recombination.

The twenty-four marker-evicted derivatives of Xc1.2 were inoculated on citrus and bean plants. They exhibited different plant reaction phenotypes, including some entirely new phenotypes, distinct from the wild type 3213 strain (Table 4-1). Five derivatives reverted back to the parental 3213 phenotype, inducing strong canker lesions on citrus (XcS1, XcS9, XcS14 and XcS15). Two derivatives caused weak canker (XcS17 and XcS20). Six derivatives elicited a previously unreported HR on grapefruit (XcS12, XcS13 and XcS24). Eleven derivatives caused no pathogenic symptoms. In contrast to wild type strain 3213, XcS5 retained the ability to elicit hyperplastic cankers on citrus, but lost the ability to elicit HR on bean, indicating that these two pleiotropic functions are separately encoded on *pthA*.

Intragenic recombination of *pthA* in *X. campestris* and *E. coli*. To demonstrate the occurrence of intragenic recombination, plasmid pUFY1.48 was introduced into three bacterial strains which carry no DNA that hybridizes with *pthA*: *X. campestris* pv. citrumelo strain 3048, *E. coli* strain DH5 α (*recA*⁻) and *E. coli* strain JM83 (*recA*⁺). The transconjugants 3048/pUFY1.48, DH5 α /pUFY1.48 or JM83/pUFY1.48 were plated on sucrose-containing medium and screened for marker-eviction. Again many marker-

evicted derivatives were obtained. The frequencies of marker-eviction of pUFY1.48 in 3048, DH5 α and JM83 were $4.05 \pm 0.44 \times 10^{-4}$, $1.06 \pm 0.13 \times 10^{-2}$ and $0.97 \pm 0.14 \times 10^{-2}$, respectively.

To examine the recombination events, plasmid DNAs were analyzed from 24 marker-evicted strains from 3048 and 14 strains from DH5 α . As shown in Fig. 4-4, different sized *Bam*HI fragments were observed, demonstrating the generation of new *pthA* homologues by intragenic recombination. Southern hybridizations of total DNAs extracted from these strains probed with the internal *Stu*I/*Hinc*II fragment of *pthA* showed only the presence of the same bands as those generated from the plasmid digestions (data not shown).

The plasmids derived from the 24 3048 (pCS series) and 14 DH5 α (pDS series) marker evicted strains were individually introduced into *X. citri* strain B21.2 (a nonpathogenic, *pthA*::Tn5-*gusA* derivative of 3213, which is virulent only on citrus), 3048 (virulent on citrus and bean) and *X. campestris* pv. *malvacearum* Xcm1003 (virulent only on cotton). The resulting transconjugants were inoculated onto citrus, bean and cotton plants (Fig. 4-5). Similar to the results observed with derivatives of Xcl.2, these *pthA* recombinants exhibited altered phenotypes and/or host specificities. For example, pCS23 conferred to B21.2 and to 3048 the ability to cause stronger canker lesions on citrus than that conferred by pZit45 (*pthA*), and a weak HR often preceeded the canker symptoms. Unlike *pthA* on pZit45, however, the recombinant gene on pCS13 conferred to B21.2 or 3048 the ability to elicit canker lesions on citrus, but not HR on bean. These results confirmed those indicated by recombinant *X. citri* mutant XcS5 (refer

Figure 4-4. A. Intragenic recombinants of *pthA* generated in *X. campestris* pv. citrumelo 3048. Plasmid DNAs were digested with *Bam*HI and fractionated in 0.7% agarose gel. Lane 1, pZit45 (*pthA*); lane 2, pUFY1.48 (*pthA::npt-sac*); lane 3-19, pCS23, pCS13, pCS16, pCS2, pCS17, pCS6, pCS1, pCS14, pCS5, pCS7, pCS11, pCS12, pCS15, pCS19, pCS21, pCS18 and pCS22, respectively. B. Intragenic recombinants of *pthA* generated in *E. coli*. Plasmid DNAs were digested with *Bam*HI and fractionated in 0.7% agarose gel. Lane 1, pZit45; lane 2, pUFY1.48; lanes 3-16, pDS2, pDS5, pDS4, pDS13, pDS1, pDS10, pDS14, pDS9, pDS3, pDS6, pDS7, pDS8, pDS11 and pDS12, respectively.

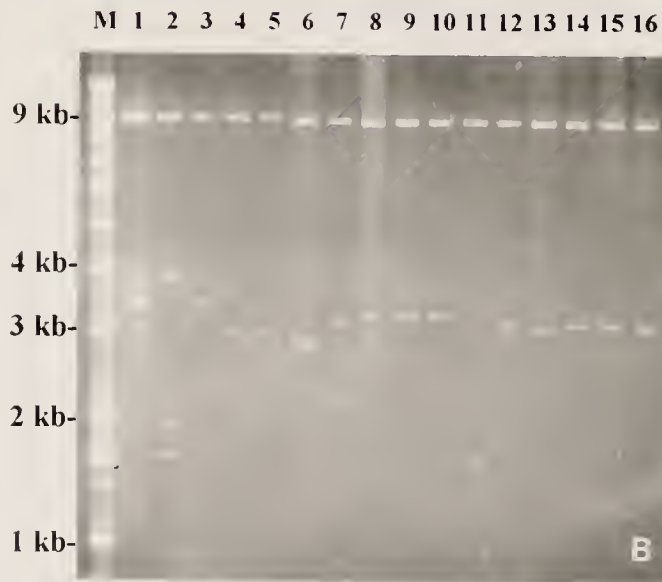
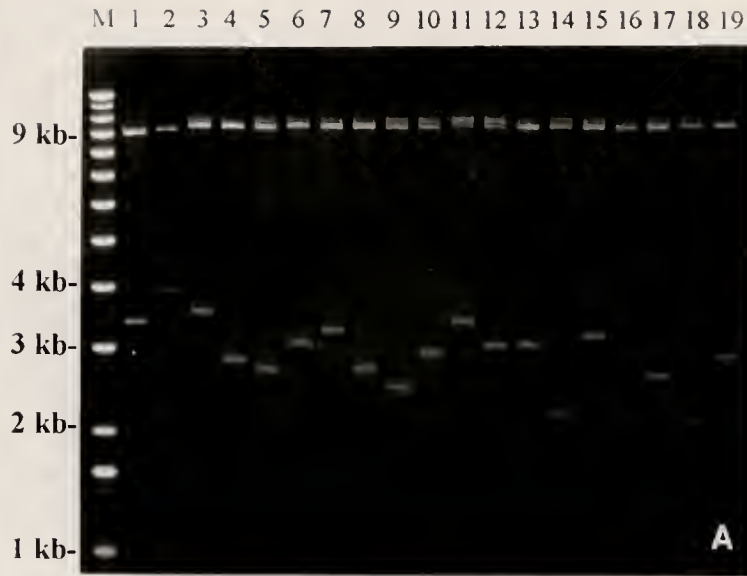
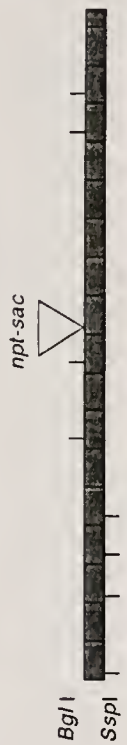


Fig. 4. Biological activity and restriction enzyme analysis of *ph4* intragenic recombinants. Only the 102bp repeated region of the gene is shown, as represented by tandemly arranged blocks. Solid blocks indicate repeats which were not likely affected by recombination. Open blocks indicate repeats which may be composite and/or located on the opposite side of the deleted region. Missing blocks represent repeats which were deleted by recombination. The *Bgl*I and *Ssp*I sites are indicated by upward and downward bars, respectively. The position of the *npt-sac* marker insertion (in the 10th repeat) in pUFY1.48 is indicated by the inverted triangle. ^a: Plasmids pCS2, pCS3, pCS7, pCS12, pCS15, pCS19, pCS20, pCS21, pDS4, pDS13 and pDS14 conferred similar phenotypes; ^b: Plasmids pCS4, pCS9, pCS10 and pDS10 conferred similar phenotypes; ^c: Plasmids pCS18, pDS6, pDS7, pDS8 and pDS11 conferred similar phenotypes. 0, no activity; C, canker lesions; mC, intermediate level canker; wC, weak canker; HR, hypersensitive reaction; wHR, weak HR; rWS, reduced watersoaking (assayed in 3048 and Xcm1003).

| Plasmid | Activity in | | |  | |
|---------------------------|---------------|------|---------|--|--|
| | B21.2 or 3048 | | Xcm1003 | | |
| | Citrus | Bean | | | |
| pUFY1.48 | 0 | 0 | 0 | | |
| pZit45 (17.5) | C | HR | rWS | | |
| pCS23 (17.5) | C/wHR | HR | rWS | | |
| pDS2 (17.5) | C | HR | rWS | | |
| pCS13 (10.5) | C | 0 | rWS | | |
| pCS16 (11.5) | mC | 0 | rWS | | |
| pCS17 (12.5) | wC | 0 | HR | | |
| pDS5 (13.5) | wC | 0 | HR | | |
| pCS5 (16.5) | wC | 0 | wHR | | |
| pCS1 (8.5) | wHR | HR | HR | | |
| pCS6 (10.5) | HR | HR | HR | | |
| pCS11 (13.5) ^a | rWS | 0 | HR | | |
| pDS9 (16.5) ^b | 0 | 0 | wHR | | |
| pCS22 (11.5) ^c | 0 | 0 | rWS | | |

above): the ability to confer hyperplastic cankers on citrus and the HR on bean are independently encoded on *pthA*.

Plasmids pCS1 and pCS6 conferred ability to elicit an HR on citrus and bean to B21.2 and 3048, and ability to elicit an HR on 9 tested cotton lines (Ac44, AcB1, AcB2, AcB4, AcB5, Acb6, Acb7, AcBIn and AcBIn3) to Xcm1003. All *pthA* derivatives tested which conferred the ability to induce HR to Xcm1003 on normally susceptible cotton line Ac44, also elicited HR on all tested cotton resistance lines. All *pthA* derivatives tested were able to confer ability to induce an HR to Xcm1003 on one or more resistance cotton lines, indicating that all *pthA* derivatives tested were functional genes.

Most of the variation within the predicted 34 amino acid repeat sequence of *pthA* occurred at positions 12 and 13, and most repeats encoded either asparagine/glycine, asparagine/isoleucine or histidine/aspartate at these positions. The latter two amino acid combinations in the *pthA* repeats could be detected at the DNA level by presence of *SspI* or *BglII* restriction sites, respectively. The *SspI* restriction enzyme cut specifically after the 36th nucleotide of the 1st, 3rd, 4th and 5th repeats of *pthA*, while the *BglII* enzyme cut specifically after the 38th nucleotide of the 7th, 9th, 15th, 16th repeats. By comparing the *BglII* and *SspI* fragments of intragenic recombinants with those of *pthA*, the specific repeats deleted or affected in 12 recombinants were determined (Fig. 4-5). Deletions involving the 7th, 8th, and 9th repeats always reduced or abolished the canker-eliciting ability of *pthA* (pCS16, pCS17, pDS5, pCS1, pCS6, pCS11, pCS22). Deletions involving the 4th, 5th, and 6th repeats abolished all avirulence specificity; these derivatives conferred ability to induce an HR on all citrus, bean and cotton lines tested.

Although recombinants with deletions in the 1st, 2nd, 16th or 17th repeats were not obtained, these results indicated that the specific order of the first nine repeats of *pthA* was important for ability to elicit cankers on citrus and for specificity of avirulence. Deletions of the 10th through the 15th repeats appeared to alter avirulence specificity.

Discussion

The complete DNA sequence of *pthA* revealed that *pthA* is highly homologous to *avrB6*, *avrBs3*, *avrBs3-2* and *avrXa10* along its entire length. The *pthA* gene encoded a protein of 1163 amino acids with calculated molecular weight of 122 kD. Western blot analyses similar to those previously published for AvrBs3 (Knoop *et al.* 1991), but using anti-PthA serum, revealed that a 122 kD protein was constitutively expressed in *X. citri* and *X. campestris* pv. *citrumelo* transconjugants containing *pthA* (refer to Chapter 5). Despite few differences in the predicted amino acid sequences of all members of the *avr/pth* gene family, *pthA* has a unique ability to induce cell divisions on citrus leaf mesophyll in addition to its pleiotropic ability to confer avirulence to pathogens of cotton and bean (Swarup *et al.* 1992). Both functions were previously found to be localized to the repeated region of *pthA* (refer to Chapter 2).

DNA sequence analysis revealed that there were five specific repeats only found in *pthA*, but not in other published members of the *avr/pth* gene family sequenced to date (*avrB6*, *avrBs3*, *avrBs3-2* and *avrXa10*). By analyzing 12 intragenic recombinants with *Bgl*II and *Ssp*I restriction enzyme digestion, we were able to approximately locate the

regions important for avirulence and pathogenic specificities. The first six repeats of *pthA* appear to be essential for the pathogenicity function and any specificity (avirulence or pathogenicity) at all. Deletions involving repeats 7 to 9 reduced or abolished canker-eliciting ability of *pthA* on citrus. Deletions involving repeats 10 to 16 determined avirulence specificity. These findings may also help to explain previously published data on *avrBs3* deletion derivatives (Herbers *et al.* 1992; Bonas *et al.* 1993). From the published data, all deletions affecting repeats 1 through 6 of *avrBs3* abolished all specificity, although not avirulence *per se*.

The role of the tandem repeats in determining pathogenic or avirulence specificity is striking, but it is not known how the repeats determine these functions. One of the important biological functions for repetitive domains in prokaryotic and eukaryotic proteins is ligand binding. In a family of clostridial and streptococcal ligand-binding protein, for example, conserved C-terminal repeat sequences function as binding sites (Wren 1991). Similarly, 34 amino acid repetitive motifs called tetratricopeptides (TPR) found in proteins encoded by many mitotic genes (e.g., CDC16, CDC23, *nuc2+* of yeast, BimA of *Aspergillus*) are implicated to pair with WD-40 repeats found in many proteins including the β -subunit of G-proteins and some transcriptional factors (Goebel and Yanagida 1991; van der Voorn and Ploegh 1992). It is possible that the repetitive domain of Pth or Avr proteins may also function as a ligand binding site, specifically interacting with corresponding plant receptors.

In this study, a *nptI-sac* cartridge was inserted into the tandemly repeated region of *pthA* to serve as a selectable marker to detect homologous recombination between

intragenic repeats. Since the production of levansucrase encoded by *sac* is lethal to gram-negative bacteria in the presence of sucrose, marker evicted strains can be selected by the *nptI-sac* marker (Ried and Collmer 1987). The regeneration of functional genes from the *pthA::nptI-sac* fragment on pUFY1.48 in 3048 or DH5 α , which contains no DNA hybridizing to *pthA*, demonstrated that intragenic recombination occurred. This was confirmed by restriction analysis of internal tandem repeats of recombinants.

Intragenic recombinants of *pthA* were generated by two possible mechanisms. The first mechanism involves a single crossover event among intragenic repeats on the same plasmid, resulting in the deletion of *nptI-sac* marker and restoration of a functional gene. The second mechanism requires a double crossover between plasmids. The frequency of *pthA* recombination in *E. coli* was observed to be *recA* independent. Strain DH5 α (*recA*) yielded recombinants at a frequency of $1.06 \pm 0.13 \times 10^{-2}$, while strain JM83 (*recA*⁺) yielded recombinants at a frequency of $0.97 \pm 0.14 \times 10^{-2}$. Since recombination involving small repeated sequences (less than 1kb) is affected only slightly by *recA* mutations (Petes and Hill 1988), recombination of *pthA* was expected to be *recA* independent.

The intragenic recombination frequencies of *pthA* observed in the *E. coli* were higher than those observed in *X. campestris* pv. citrumelo ($4.05 \pm 0.44 \times 10^{-4}$) and *X. citri* ($5.22 \pm 1.8 \times 10^{-5}$). This may be due to differences in the copy number of plasmids in which intragenic recombination occurred. Recombination of *pthA* in *X. citri* strain Xc1.2 involved a native plasmid, while recombination in *E. coli* and *X. campestris* pv. citrumelo involved pUFY1.48, which appeared (based on our plasmid DNA

extractions) to replicate at a higher copy number in *E. coli* than in *Xanthomonas*. However, since we did not determine plasmid copy number, these results may be due to different genetic backgrounds in each strain.

In animal pathogens, some pathogenicity genes encode surface proteins with a distinctive arrangement of tandem repeats which may provide a protective, strain-specific conformational epitope for evasion of host immunity (Hoft *et al.* 1989; McConkey *et al.* 1990; Hollingshead *et al.* 1987). Intragenic recombination between homologous repeats has been found to be responsible for antigenic variations in the pathogens such as *Streptococcus*, *Neisseria*, *Salmonella*, *Plasmodium* and *Trypanosoma* (McConkey *et al.* 1990; Hollingshead *et al.* 1987; Hagblom *et al.* 1985; Frankel *et al.* 1989). The resulting antigenic variability allows pathogens to escape the host immune response. In this study, we demonstrated that intragenic recombination of a single plant bacterial gene can: 1) alter host specificity, 2) generate new pathogenic phenotypes, and 3) evade host plant defenses. The evolutionary success of many plant pathogens may rely on their ability to avoid host recognition. Tandemly repeated motifs in *pthA* and other members of the gene family provide hot spots for recombination, and thus may accelerate evolution of this gene family. Intragenic recombination between inexact repeats not only introduces genetic variation, but also has the potential to amplify mutagenic effects. As shown in the results, homologous recombination can lead to either duplication or deletion of repeat blocks. A mechanism to generate genetic variation at high frequency may also have the negative side effect of generating unnecessary, gratuitous *avr* genes.

Previously reported natural mechanisms for race change included deletions (De Feyter *et al.* 1993), point mutations (Kobayashi *et al.* 1990; Joosten *et al.* 1994) or transposon inactivation (Kearney *et al.* 1988) of *avr* genes and always resulted in a loss of avirulence on a specific host plant, but not gains of specific avirulence. Because of the unique 102bp tandem repeats and their role in determining both pathogenic and avirulence specificities, intragenic recombination of *pthA* not only caused the loss of pathogenic or avirulence phenotypes on a specific host, but also simultaneously resulted in the gain of new pathogenic phenotypes and avirulence specificities. Many intragenic recombinants of *pthA* lost the ability to confer canker-like lesions on citrus to 3048, but conferred a new phenotype by reducing the watersoaking symptoms of 3048. Some intragenic recombinants lost the ability to confer cankers on citrus and instead elicited an HR on citrus, indicating that normnsensitive (canker) and hypersensitive reactions may share a very similar signal transduction pathway as initially suggested by Klement (1982). Other recombinants lost the ability to elicit HR on bean, but retained the ability to induce cankers on citrus. Therefore, the pleiotropic pathogenic and avirulence functions of *pthA* were separated by the rearrangement of tandem repeats through intragenic recombination.

Members of this *avr/pth* gene family have been found in many *Xanthomonas* species and pathvars. When present in appropriate xanthomonads, many are able to elicit an HR on diverse plant species including cotton, citrus, bean, tomato, pepper and rice. As shown in Fig. 4-5, one intragenic recombinant of *pthA* (pCS6) has the ability to elicit a strong HR on plants from taxonomically distinct plant families such as *Rutaceae* (citrus), *Leguminosae* (bean) and *Malvaceae* (cotton), suggesting that the corresponding

resistance genes may be widespread in different plant species. Tomato disease resistance gene *Pto*, which encodes a protein kinase, hybridizes with multiple DNA fragments found in a variety of different plants, indicating the presence of *Pto* homologues (Martin *et al.* 1993). During the evolutionary process, members of the *avr/pth* gene family might have moved horizontally among diverse xanthomonads pathogenic to a wide range of plants (Gabriel *et al.* 1993; De Feyter *et al.* 1993), thereby enabling interactions with members of the same plant gene family in a wide range of plants. As a result, members of this *avr/pth* gene family may have accelerated the evolution and diversity of the corresponding plant *R* gene family.

The gene-for-gene concept is a major model for research on the reciprocal evolution between plants and parasites (Thompson and Burdon 1992). In the pathogen, avirulence genes may be mutated to overcome resistance by evading host recognition. In the plant, resistance genes may also evolve to recognize new avirulence genes, resulting in new resistance genes. Recent genetic mapping data have shown that disease resistance loci (especially those corresponding to different races of the same pathogen) are often clustered within small genetic intervals, suggesting that intralocus recombination may be responsible for generating resistance specific for new races of plant pathogens (Dickinson *et al.* 1993; Martin *et al.* 1993; Pryor 1987). Intragenic recombination of *pthA* (a member of an *avr* gene family) demonstrated in this research provides compelling evidence that intragenic and intralocus recombination may play an important role during the reciprocal evolution of gene-for-gene interactions.

CHAPTER 5

PLANT NUCLEAR TARGETING SIGNALS ENCODED BY A FAMILY *XANTHOMONAS* AVIRULENCE/PATHOGENICITY GENES

Introduction

Interactions of microbial plant pathogens carrying avirulence (*avr*) genes with host plants carrying specific resistance (*R*) genes results in plant defense responses, often observed as a hypersensitive reaction (HR) that is characterized by rapid plant cell death at the site of infection. Several models have been proposed to explain the genetically specific recognition between microbial *avr* genes and plant *R* genes in these gene-for-gene interaction (Gabriel and Rolfe 1990), and different models may be applicable to different systems. In the elicitor/receptor model, *avr* genes encode a low molecular weight signal molecule (elicitor) which is perceived by a sensor encoded by the corresponding *R* genes and results in an HR. In *Pseudomonas syringae* pv. tomato, *avrD* encodes an enzyme involved in the synthesis of a low molecular weight glycolipid elicitor (Midland *et al.* 1993; Smith *et al.* 1993). This elicitor induces an HR specifically on soybean plants containing the resistance gene *Rpg4* (Keen *et al.* 1990; Keen and Buzzel 1991; Kobayashi *et al.* 1990). An alternative model is the dimer hypothesis in which the protein product of an *avr* gene interacts directly with the protein product of a plant *R* gene or with the *R* gene itself (Ellingboe 1982). The extracellular peptide encoded by *avr4* and *avr9* of

the fungal pathogen *Cladosporium fulvum* can directly induce a hypersensitive response (HR) on tomato cultivars carrying the resistance gene *Cf4* or *Cf9* (Joosten *et al.* 1994; van Kan *et al.* 1991). However, other than these three examples, the microbial signal molecules that determine avirulence remain unidentified. In all cases, it is unknown how the signals encoded by *avr* genes are specifically recognized by host cells with *R* genes, triggering plant defense responses.

The first plant *R* gene, isolated from tomato, was shown to encode a serine/threonine protein kinase that is homologous to the receptor protein kinase involved in *Brassica* pollen-stigma recognition and to the Raf protein kinase involved in the mammalian Ras signaling pathway (Martin *et al.* 1993). Since transcriptional activation of plant defense genes is modulated by phosphorylation (Felix *et al.* 1991; Yu *et al.* 1993), and can be blocked by inhibitors of mammalian protein kinases (Raz and Fluhr 1993), it was proposed that recognition of *avr* signals by *R* gene products triggers phosphorylation cascades, leading to plant defense responses (Lamb 1994).

In mammalian systems, two nearly complete signal transduction pathways used by growth factors and/or cytokines have been elucidated (Culotta and Koshland 1993). The best known is the Ras pathway, centered around the protein product of the prototype oncogene *ras* (Hall 1993, Schlessinger 1993). Upon binding to extracellular signals, the activated receptor protein kinase transduces signals to the GRB2, SOS, Ras and Raf proteins. Raf (a Pto homolog) then initiates the mitogen-activated protein (MAP) kinase cascade (Crews and Erikson 1993; Nishida and Gotoh 1993), which brings protein kinases into the nucleus, activating nuclear transcriptional factors. The other route from

membrane to nucleus is more direct. In these cases, tyrosine kinases pick up signals from receptors in the membrane and activate transcription factor subunits in the cytoplasm, which are transported into the nucleus and induce transcription (Hunter 1993, Kishimoto *et al.* 1994). In both cases, activated protein kinases and/or transcriptional factors need to enter the nucleus in order to induce gene expression which may lead to different physiological outcomes, including oncogenesis and apoptosis.

Viral pathogens encode some transcriptional factors that can directly enter the nucleus, modulate gene expression of host cells. The bacterial plant pathogen *Agrobacterium tumefaciens* also encodes several proteins targeted to the plant cell nucleus (Citovsky and Zambryski 1993). Transport of proteins into the nucleus is an active process and requires that proteins contain suitable nuclear localization sequences (NLSs, Nigg *et al* 1991). NLSs are usually short stretches of 8-10 amino acids characterized by basic amino acid residues and proline. NLSs are retained in the mature protein, may be located at any position as long as they exposed on the protein surface, and can be present in multiple copies. NLSs are recognized by the receptor, called NLS-binding proteins, which direct NLS-containing proteins into nucleus through nuclear pores (Silver 1991). NLSs from different organisms, including the prokaryote *Agrobacterium*, are functional throughout the eukaryotic kingdom. Most of the characterized NLS-containing proteins were reported from mammalian virus and animal cells. Many are oncogene products, hormone receptors and transcriptional factors, including those involving later steps of the Ras signaling pathway such as the transcriptional factor AP-1 (Jun/Fos) (Kerr *et al.* 1992). A few NLS-containing proteins from *Agrobacterium*, plant and plant virus

have also been characterized (Citovsky and Zambryski 1993; Restrepo *et al.* 1990; van der Krol and Chua 1991; Varagona *et al.*, 1991, 1992). To the best of my knowledge, however, NLSs encoded by *avr* genes of plant pathogens have not been reported.

Recently, a large family of avirulence (*avr*)/pathogenicity (*pth*) genes has been identified in many species and pathovars of *Xanthomonas*, a major group of plant bacterial pathogens. Members includes *avrBs3*, *avrBs3-2* and *avrBsP* of *X. campestris* pv. *vesicatoria* (Bonas *et al.* 1989, 1993; Canteros *et al.* 1991), *avrB4*, *avrb6*, *avrb7*, *avrBln*, *avrB101*, and *avrB102* of *X. c.* pv. *malvacearum* (De Feyter and Gabriel 1991; De Feyter *et al.* 1993), *pthA* of *X. citri* (Swarup *et al.* 1991, 1992), and *avrxa5*, *avrXa7* and *avrXa10* of *X. oryzae* pv. *oryzae* (Hopkin *et al.* 1992). This *avr/pth* gene family comprises the majority of described *Xanthomonas avr* genes and constitutes a large portion of all *avr* genes cloned to date (Gabriel *et al.* 1993). The *pthA* gene of *X. citri* and *avrb6* of *X. c.* pv. *malvacearum* are particularly intriguing because of their pathogenicity functions (Swarup *et al.* 1992, Chapter 2). Gene *pthA* is essential for *X. citri* to induce cell division specifically on citrus plants leading to hyperplastic canker lesions. Gene *avrb6* is important for *X. c.* pv. *malvacearum* to induce watersoaking symptoms specifically on cotton. These two genes also function pleiotropically as *avr* genes.

Despite the diverse avirulence and pathogenic specificities encoded by these *avr/pth* genes, their DNA sequences are nearly identical (95-98%) (De Feyter *et al.*, 1993, Chapter 4). Both pathogenic and avirulence specificities of the *avr/pth* genes are determined by the nearly identical, 34 amino acid tandem repeats encoded in the central

region of the genes (Herbers *et al.* 1992; Chapter 2). To account for the precise recognition and diverse specificities, it has been speculated that the *avr/pth* protein product may directly mediate the plant hypersensitive response (Herbers *et al.* 1992), as initially suggested in the dimer hypothesis.

In this study, we demonstrated that *pthA* conferred to *X. campestris* pv. *malvacearum* (which is nonpathogenic on citrus) the ability to elicit hyperplastic canker symptoms on citrus, independently of bacterial growth *in planta*. Although the proteinaceous elicitors encoded by *pthA* or *avrb6* were not detected, three nuclear localization sequences were identified to be encoded by all members of the *Xanthomonas avr/pth* gene family sequenced to date. Furthermore, the C-termini of PthA and Avrb6, containing the NLSs were shown to be capable of directing β -glucuronidase into the nuclei of plant cells.

Materials and Methods

Bacterial strains and plasmids. *Escherichia coli* strains used were DH5 α (Gibco-BRL, Gaithersburg, MD) and BL21(DE3)pLysS (Novagen, Madison, WI). *Xanthomonas* strains used were *X. citri* 3213 (causes hyperplastic cankers on citrus, Swarup *et al.* 1991, 1992), its marker exchange mutant Xc1.2 (*pthA::npt-sac*, causes no symptom on citrus), *X. campestris* pv. *citrumelo* 3048 (causes water-soaked leaf spots on citrus and common bean, Swarup *et al.* 1991, 1992), *X. campestris* pv. *malvacearum* XcmH1005 (causes water-soaked leaf spots on cotton) and its mutants XcmH1407 (*avrb6::Tn5-gusA*,

refer to Chapter 2), HM2.2S (seven *avr* genes deleted, refer to Chapter 3). *E. coli* strains were grown in Luria-Bertani (LB) medium (Sambrook *et al.* 1989) at 37°C or 30°C. *Xanthomonas* strains were grown in PYGM (peptone-yeast extract-glycerol-MOPS) medium at 30°C as described (De Feyter *et al.* 1990). Plasmids pUC19 (Yanisch-Perron *et al.* 1985) and pUFR047 (De Feyter *et al.* 1993) were used as cloning vectors. Plasmid pUFR127 (De Feyter and Gabriel 1991a) contains *avrb6* on a 5-kb DNA fragment. Plasmid pZit45 (Swarup *et al.* 1992) contains *pthA* on a 4.5-kb DNA fragment. Plasmid pUFY20 contains a chimeric gene consisting of the 5' and 3' regions of *avrb6* and internal repeated region of *pthA* (refer to Chapter 2). Plasmids pCS6 and pCS23 contain intragenic recombinants of *pthA* (refer to Chapter 4). Triparental matings were carried out to transfer plasmids from *E. coli* DH5 α to *Xanthomonas* strains by using pRK2013 or pRK2073 as helper plasmids (Swarup *et al.* 1991, De Feyter and Gabriel 1991a). To transfer plasmids into *X. campestris* pv. *malvacearum* strains, the modifier plasmid pUFY054 carrying *XcmI* and *XcmIII* methylase genes was used to increase the transfer frequency (De Feyter and Gabriel 1991b).

Preparation of potential elicitors and plant inoculations. To detect elicitor activity, crude lysates were prepared as described (He *et al.* 1993) from *Xanthomonas* and *E. coli* cells carrying *pthA* or its derivatives. Basically, bacterial cultures were grown overnight, harvested by centrifugation and resuspended in 10mM Tris-HCl (pH8.0) solution at an OD₆₀₀ of 0.5-1. Bacterial cells were lysed by lysozyme treatment (2mg/ml lysozyme in 10mM Tris-HCl, pH8.0) or disrupted by sonication in the presence of 1 mM PMSF (phenylmethylsulfonyl fluoride). PthA protein was also

overexpressed in *E. coli* cells and purified by affinity chromatography (described later). Cell-free suspensions (in 10mM Tris-HCl, pH8.0) or affinity column purified PthA were immediately infiltrated into leaves of citrus and cotton plants.

Citrus (*Citrus paradisi* 'Duncan', grapefruit) and cotton (*Gossypium hirsutum*) plants were grown under greenhouse conditions. Cotton lines used were Acala-44 (Ac44) and its congenic resistance lines Acb5b, Acb6, AcBIn3 as described (De Feyter *et al.* 1993; Swarup *et al.* 1992). *X. campestris* pv. *malvacearum* transconjugants carrying *pthA* or its derivatives were inoculated into leaves of citrus plants in BL-3P level containment (refer to Federal Register Vol.52, No. 154,1987) at the Division of Plant Industry, Florida Department of Agriculture, Gainesville.

Preparation of antisera and immunoblotting. A 3.7kb *Bam*HI fragment carrying intact *pthA* was generated from partial digestion of pZit45 (Swarup *et al.* 1992), and cloned into expression vector pET-19b (Novagen, Madison, WI). The fusion sites of the resulting construct pUFY50.13 were determined by DNA sequence analysis. PthA protein was overexpressed in *E. coli* strain BL21(DE3)/pLysS containing pUFY50.13 in the presence of 1mM IPTG (isopropyl- β -D-thiogalactopyranoside). Since proteins expressed on pET-19b are designed to contain 10 histidine residues at the amino terminus as an affinity handle, overexpressed PthA was readily purified by His-tag affinity chromatography (Novagen, Madison, WI). For immunization of rabbits, PthA was further purified by SDS-polyacrylamide gel electrophoresis. Antiserum against PthA was prepared from a New Zealand rabbit by Cocalico Biologicals, Inc. (Reamstown, PA). For immunoblotting, *E. coli* or *Xanthomonas* cells were harvested by centrifugation and

resuspended in sample buffer (50 mM Tris-HCl, pH6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue). After samples were boiled for 2 min and centrifuged for 5 min, they were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose by electroblotting (Towbin *et al.* 1979). Western blots were probed with anti-PthA antibody and visualized with goat anti-rabbit antibody conjugated with alkaline phosphatase.

DNA constructs for GUS fusion proteins. The DNA coding sequences for the C-terminal region (191 amino acids) of PthA and Avrb6 were amplified by PCR from the plasmid pZit45 (Swarup *et al.* 1992) by using the following primers:

1. CTCTAGAGCCATGACGCAGTTC; 2. CAGATCTCTGAGGCAATAGCTC.

The amplified fragments were first cloned into pGEM-T (Promega Inc. Madison, WI), then cut out with *Xba*I/*Bgl*II and recloned into the *Xba*I/*Bam*HI site of plant expression vector pBI221 (Clontech, CA). The resulting plasmids pUFY082 and pUFY083 contain the chimeric gene with the C-terminal coding sequence of PthA and Avrb6, respectively, translationally fused with 5' end of GUS gene. Both pUFY082 and pUFY083 were checked by sequence analysis at the fusion site.

Onion transformation system. The Helium Biolistic gene transformation system (Du Pont) was used to transform epidermal cell layers of white onion as described (Varagona *et al.* 1992). Inner epidermal layers were peeled and placed inside up on Petri dishes containing MS (Murashige and Skoog 1962) basal media (4.4 g/L MS salt mixture, Sigma, M5519, 30 g/L sucrose, pH 5.7) with 2.5 mg/L amphotericin B (Sigma) and 6% agar. DNA samples were prepared as described (Taylor and Vasil 1991). Five μ g of column-purified (Magic Prep, Promega) plasmid DNA was precipitated onto 1 mg

of 1.6 μm gold particles using 50 μL of 2.5 M CaCl_2 and 10 μL of 100 mM spermidine. DNA-coated gold particles were washed with 50 μl of 70% and 100% ethanol and finally resuspended in 60 μL of 100% ethanol. The samples were dispersed by submerging in a waterbath sonicator for a few seconds. Five μL samples were pipetted onto particle delivery discs and delivered into onion epidermal cell layers at pressures of 1100 or 1300 p.s.i.. After microprojectile bombardment, Petri dishes were sealed with parafilm and incubated overnight at 28°C in the dark. The assays for nuclear localization activity of the fusion protein were repeated in three independent transformation tests.

Histochemical analysis. The histochemical GUS assay was used to determine the location of GUS fusion proteins in onion cells as described previously (Jefferson, 1987, Varagona et al 1992). Onion epidermal layers were incubated at room temperature in X-glu solution (50 mM phosphate buffer, pH7.0, 1mM EDTA, 0.001% Triton X-100. 0.05mM sodium ferricyanide and ferrocyanide, 2mM X-glu). After detection of blue color, onion epidermal layers were mounted on a glass slide with a solution containing 20 $\mu\text{g/ml}$ of the nucleus-specific dye 4',6-diamidino-2-phenylindole (DAPI, 0.1 X phosphate buffer, 10mM sodium azide, 90% glycerol). Cellular location of the blue indigo dye produced by oxidative dimerization of the GUS product was determined under bright-field optics using a Zeiss Axiophot microscope and compared with the location of DAPI-stained nuclei under fluorescence optics.

Results

Citrus canker symptoms elicited by *X. campestris* pv. *malvacearum* carrying *pthA*. Gene *pthA* is required for pathogenicity of *X. citri* on citrus and confers canker-eliciting ability to many citrus-compatible xanthomonads (Swarup *et al.* 1991). In this study, *pthA* and its derivatives were introduced into *X. campestris* pv. *malvacearum*, a cotton pathogen that does not grow on citrus plants (Kingsley *et al.* 1993). When inoculated on citrus, transconjugants of *X. c.* pv. *malvacearum* carrying *pthA* elicited hyperplastic canker-like lesions on citrus (Fig. 5-1). The transconjugant XcmH1407/pUFY20, which differs from XcmH only by replacing the repeated region of *avrb6* with that of *pthA*, also elicited canker-like lesions. However, *pthA* did not enable *X. c.* pv. *malvacearum* to grow on nonhost citrus, and high initial inoculum levels ($> 10^7$ bacteria/ml) were required to elicit visible canker-like symptoms.

Attempt to detect elicitor activity encoded by *pthA*. The diverse specificities encoded by tandem repeats and the fact that a few amino acid changes in the tandem repeats of *pthA* enabled a cotton pathogen to elicit canker symptoms on nonhost citrus suggest that *pthA* and other members of the gene family may encode protein elicitors directly interacting with plant receptors. In repeated attempts, however, cell-free lysates and affinity column purified PthA protein from *X. campestris* pv. *malvacearum* and/or *E. coli* cells carrying *pthA* or its derivatives failed to elicit canker or HR symptoms on citrus and cotton plants.



Figure 5-1. Canker symptoms elicited by *X. campestris* pv. *malvacearum* transconjugants on nonhost citrus. 1, XcmH/pUFR047; 2, XcmH1407/pUFR047; 3, XcmHM2.2S/pUFR047; 4, XcmH/pZit45; 5, XcmH1407/pUFY20; 6, XcmHM2.2S/pZit45.

Constitutive expression of PthA protein. Western blot analysis revealed that the predicted 122 kD PthA protein was constitutively expressed in *Xanthomonas* cells carrying *pthA* (Fig. 5-2). The antibody specifically recognized 3-4 proteins in protein extracts of wild type *X. citri* strain 3213, which contains *pthA* plus three putative homologues. As predicted by the size of these homologous gene, the largest protein was PthA (indicated by an arrow in lane 1, Fig. 5-2). This was confirmed by Western analysis of the marker exchange mutant Xc1.2 (*pthA::npt-sac*). Gene *pthA* was disrupted in Xc1.2 by a *nptI-sac* marker inserted at the 10th tandem repeat (Chapter 4), which was expected to generate a truncated protein of 68kD upon translation. As shown in lane 2, Fig. 5-2, this mutant lost the PthA protein and instead expressed a protein close to the size of predicted truncated protein (68 kD). The anti-PthA antibody did not react with extracts from *X. campestris* pv. *citrumelo* 3048 which contains no genes homologous to *pthA*. However, PthA protein was constitutively expressed in rich medium by the 3048 transconjugant carrying *pthA* (indicated by an arrow in lane 4, Fig. 5-2).

The C-terminal region of Avr/Pth proteins contain nuclear localization consensus sequences. The proteins encoded by all sequenced members of the *Xanthomonas avr/pth* such as *avrb6* (De Feyter *et al.* 1993), *pthA* (chapter 4), *avrBs3* (Bonas *et al.* 1989, 1993) and *avrXa10* (Hopkins *et al.* 1992) are highly homologous and only vary in the internal repetitive region which determines avirulence and pathogenic specificities. Analysis of the amino acid sequences encoded by these genes revealed the presence of three stretches of basic residues with complete homology with the nuclear localization

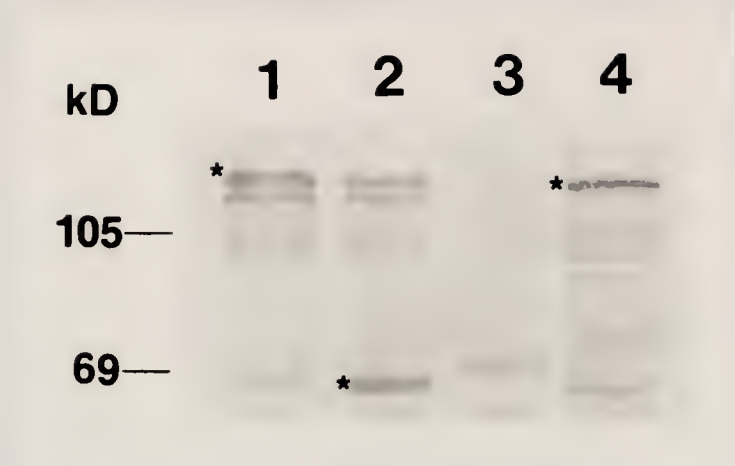


Figure 5-2. Western blot analysis of protein extracts from *Xanthomonas* strains. 1, *Xanthomonas citri* strain 3213; 2, marker exchange mutant Xc1.2 (*pthA::npt-sac*); 3, *X. c. pv. citrumelo* strain 3048; 4, 3048 transconjugant carrying pZit45 (*pthA*). Asterisk indicate the band of PthA protein or its truncated derivative.

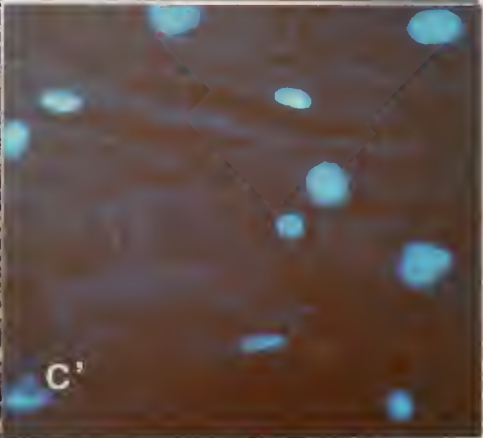
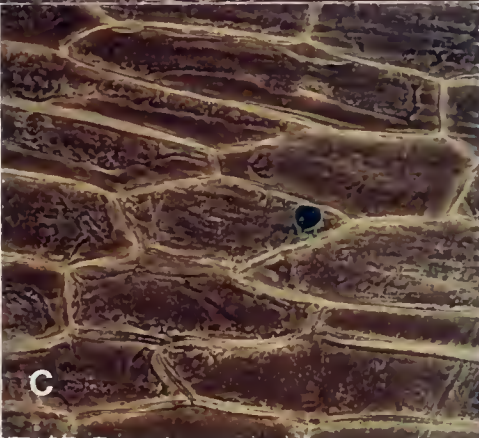
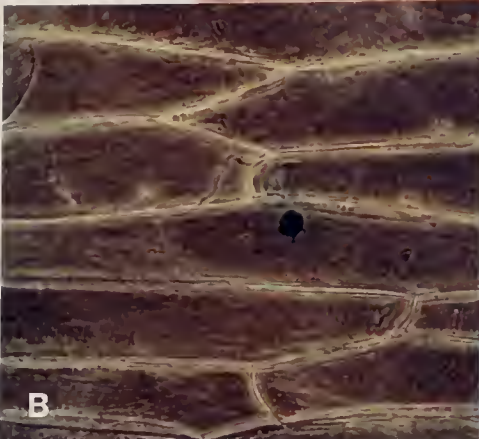
| <u>Protein</u> | <u>Nuclear Localization Sequences</u> |
|---------------------------|---|
| Consensus sequence | K R/K X K/R |
| SV40-T (monopartite) | p K K K R K v |
| Nucleoplasmin (bipartite) | K R p a a t K K a g g a K K K K l |
| VirD2 | K R p R e d d d g e p s e R K R e R d |
| PthA and Avrb6 | K R a K p-40 aa-R K R s R-31 aa-R v K R p R |

Figure 5-3. Nuclear localization sequences from SV40 (T-antigen), *Xenopus* (nucleoplasmin), *Agrobacterium* (VirD2) and *Xanthomonas* (PthA and Avrb6). Capital letters denotes basic residues presumed important for nuclear targeting.

consensus sequences (K-R/K-X-R/K) found in many characterized nuclear localized proteins (Chelsky *et al.* 1989) (Fig. 5-3). These three putative nuclear localization sequences (NLSs) are located at the carboxyl-terminal part of the proteins, at positions 1020-1024 (K-R-A-R-P), 1065-1069 (R-K-R-S-R), and 1101-1106 (R-V-K-R-P-R) in PthA. The first and third putative NLSs also contains proline residues which are often found in NLSs. In addition, three putative casein kinase II sites were found in proximity to these three nuclear localization consensus sequences. They are located at positions 994-997 (TELE), 1068-1071 (SRSD), 1120-1123 (TAAD) in PthA. This feature is common to many nuclear localized proteins which often have casein kinase II sites (S/T-X-X-D/E) at a distance of 10-30 amino acids up or downstream from the NLSs (Rihs *et al.* 1991).

The C-terminus of PthA and Avrb6 proteins directed β -glucuronidase into the plant nucleus. The 68 kD β -glucuronidase (GUS) protein has been shown to localize in the cytoplasm (Restrepo *et al.* 1990; van der Krol and Chua 1991) and is too large to enter the nucleus without an NLS (Varagona *et al.* 1992). As shown in Fig. 5-4A, when pBI221 (encoding GUS) was introduced into onion epidermal cells, GUS activity was found in the cytoplasm and not in the nucleus. To determine whether or not the putative NLSs are functional in plant cells, the DNA coding sequences for the C-terminal region (191 amino acids) of PthA and Avrb6 were translationally fused with the 5' end of a GUS reporter gene. The resulting constructs were introduced into onion epidermal cells by microprojectile bombardment and expressed transiently. Intracellular localization of GUS fusion proteins (approximately 90-kD) containing the C-terminal region of PthA

Figure 5-4. Histochemical localization of GUS and GUS fusion proteins containing the C-terminal region of PthA or Avrb6 in transformed onion epidermal tissues. Tissue were simultaneously analyzed using X-glu staining (A, B, C) and nuclei-specific DAPI staining (B', C'). A, transformed with pBI221 encoding GUS protein; B and B', transformed with pUFY082 encoding GUS fusion protein containing the C-terminus of PthA; C and C', transformed with pUFY083 encoding GUS fusion protein containing the C-terminus of Avrb6.



or Avr_{b6} was determined histochemically, with GUS activity localized specifically to the nucleus of plant cells (Fig. 5-4 B and C). In repeated experiments, more than one hundred nuclei were observed to be GUS positive. Rarely, some diffuse GUS activity was observed within the cytoplasm. The location of nuclei was confirmed by staining with the nucleus-specific dye 4',6-diamidino-2-phenylindole (DAPI) and observed under fluorescence microscope (Fig. 5-4 B' and C').

Discussion

The pathogenicity gene, *pthA*, is required for *X. citri* to elicit hyperplastic canker symptoms on citrus and confers canker-eliciting ability to several xanthomonads that are mildly pathogenic on citrus (Swarup *et al.* 1991). *X. c. pv. malvacearum*, a cotton pathogen, is nonpathogenic on citrus and can not grow on citrus plants (Kingsley *et al.* 1993), but it has high homology (90%) with *X. citri* as shown by DNA reassociation (Egel *et al.* 1991). In this study, introduction of *pthA* into *X. campestris* pv. *malvacearum* strain XcmH1005, or the replacement of the tandem repeats of *avrb6* in XcmH1005 with those of *pthA*, conferred the ability to induce hyperplastic canker lesions on citrus. The host-specific canker symptoms induced by *X. campestris* pv. *malvacearum* transconjugants carrying *pthA* were independent of bacterial growth *in planta* since high inoculum levels ($> 10^7$ cells/ml) were required to elicit visible symptoms. Therefore, additional virulence genes are needed to extend the host range of *X. campestris* pv. *malvacearum* to include citrus plants. Gene *pthA* belongs to a *Xanthomonas avr/pth* gene

family and differs only slightly from other members in the repeated region of genes (Swarup *et al.* 1992; also refer to Chapter 4). It is striking that only a few amino acid changes in the tandem repeats of a single *avr* gene enabled XcmH1005 to elicit canker symptoms on citrus. Since the repetitive domains of prokaryotic and eukaryotic proteins often function as ligand binding sites and determine specificity (Wren 1991; McConkey *et al.* 1990), *pthA* may encode a proteinaceous elicitor capable of interacting with a plant receptor and inducing host-specific symptoms.

Despite repeated attempts, elicitor activity was not detected in cell-free crude extracts from *X. c. pv. malvacearum* and *E. coli* cells carrying *pthA* or its derivatives. Affinity column purified PthA also failed to elicit pathogenic symptoms. The failure could result from a variety of potential problems. Protein elicitors may be unstable and may need post-translational processing. Protein elicitors may not be able to gain entry into plant cell in the absence of living pathogens which secrete extracellular enzymes and weaken plant cell wall.

Western blot analysis revealed that the predicted 122 kD PthA protein was constitutively expressed in *Xanthomonas* cells carrying *pthA*. AvrBs3, a homolog of PthA, is also constitutively expressed in *X. campestris* pv. *vesicatoria* grown on rich media (Knoop *et al.* 1991). Despite this constitutive expression, *hrp* (hypersensitive response and pathogenicity) gene function is absolutely required for *Xanthomonas* carrying *avrBs3* to elicit an HR on pepper (Knoop *et al.* 1991) and for *Xanthomonas* carrying *pthA* to elicit cankers on citrus (unpublished data, Gabriel). Since *hrp* genes are known to be involved in the export of proteinaceous HR elicitors (He *et al.* 1993), and

since *hrp*⁻ mutations abolish activity of two members of the gene family, it seems likely that the proteins encoded by these *avr/pth* genes are exported from the bacteria in their interactions with plants (Fenslau 1993).

No direct evidence has been found for the export of proteins encoded by this gene family. Cell fractionation indicates that the major portion of AvrBs3 protein is in the bacterial cytoplasm and immunoelectron microscopy fails to detect AvrBs3 in the bacterial outer membrane or evidence of secretion (Knoop *et al.* 1991; Brown *et al.* 1993). The failure to detect protein secretion could have resulted from protein instability or very low levels of the antigen in the extracellular environment. For example, VirE2 of *Agrobacterium tumefaciens* was localized in the bacterial cytoplasm by cell fractionation and by immunoelectron microscopy (Christie *et al.* 1988; Thorstenson and Zambryski 1994), however, it is known to be exported together with T-DNA and enters the nucleus of plant cells (Citovsky *et al.* 1989, 1992)

The translocation of the *Agrobacterium* T-DNA complex into the nucleus of plant cells can be viewed as an unique signaling pathway (Citovsky and Zambryski 1993). The T-DNA is coated by the single strand binding protein VirE2 and piloted by protein VirD2 (Christie *et al.* 1988; Ward and Barnes 1988). Both VirE2 and VirD2 contain nuclear localization sequences (NLSs) which could facilitate the translocation of T-DNA complex into the nucleus (Citovsky *et al.* 1992; Howard *et al.* 1992; Shurvinton *et al.* 1992; Tinland *et al.* 1992). Nuclear localization sequences are short stretches of basic amino acid residues conserved in many nuclear localized proteins. VirD2 and VirE2 are the only bacterial proteins that have been reported to contain nuclear localization sequences.

Analyses of the predicted amino acid sequences of PthA and its homologs encoded by members of the *Xanthomonas avr/pth* gene family revealed three putative nuclear localization sequences located in the C-terminal region of these proteins. By transiently expressing GUS fusion proteins in onion epidermal cells, the C-terminal region of PthA or Avr6 was shown to direct the GUS fusion protein into the nucleus of plant cells. Therefore, functional nuclear localization sequences were identified in the proteins encoded by members of the *Xanthomonas avr/pth* gene family.

We have examined amino acid sequences encoded by some other *avr* genes, and at least one of them, AVR4 of *Cladosporium fulvum* (Joosten *et al.* 1994), also contains a putative bipartite nuclear localization sequence (KKWCDYPNLSTCPVKTPGPKPKK) in the C-terminal region. AVR4 and AVR9 of *Cladosporium fulvum* are the only known race-specific protein encoded by *avr* genes that are capable of directly eliciting an HR on plants. It will be interesting to see if this putative NLS are functional in plant cells.

Changes in the cell division and differentiation status of eukaryotic cells may be accompanied by the selective relocalization of proteins between cytoplasm and nucleus (Nigg *et al.* 1991). This holds for *fos* and *rel* encoded oncoproteins, certain transcriptional factors, steroid hormone receptors, the *Drosophila* morphogen Dorsal and the *Agrobacterium* T-complex. For example, the activity of the Rel/NF κ B/Dorsal family of proteins is controlled by sequestration in the cytoplasm in association with inhibitory proteins called I κ B (Kerr *et al.* 1992). In unstimulated cells, transcription factor NF κ B is associated with I κ B and retained in the cytoplasm. However, external signals activate protein kinases which phosphorylate I κ B, resulting in the dissociation of the NF κ B-I κ B

complex. Subsequent translocation of NF κ B into the nucleus triggers gene expression. In mammalian systems, NF κ B is activated by oxygen radicals induced by diverse signals through distinct intracellular pathways (Schreck *et al.* 1991). In plants, active oxygen species are known to be involved in hypersensitive response and activation of plant defense genes (Bradley *et al.* 1992; Chen *et al.* 1993; Legendre *et al.* 1992). However, plant homologues of NF κ B and I κ B are not known. The inhibitory I κ B proteins contain 5 to 8 ankyrin repeats. The ankyrin repeat motif is a 33 amino acid unit that is found in a number of transcription factors, receptors and ion transporters and is important in protein-protein interactions (Blank *et al.* 1992; Sentenac *et al.* 1992). Interestingly, two short stretches of conserved amino acids (G-X-T-P and V-X-X-L-L) found in the ankyrin repeat also exist in the 34 amino acid repeats of PthA and its homologs. However, it is not known whether this is just coincidental or has functional meaning.

Based on the known data and previous models (Gabriel and Rolfe 1990), a testable hypothesis was proposed to explain the possible mechanism by which specific signals encoded by the *Xanthomonas avr/pth* gene are perceived and transduced (Fig. 5-5). After entering intercellular space of plant tissues, the pathogen releases the Avr/Pth proteins by an *hrp*-dependent export pathway activated by plant signals (Fenselau *et al.* 1992). At the same time, the pathogen also secretes extracellular enzymes to weaken the plant cell wall. Avr/Pth proteins enter plant cell through a process similar to the receptor-mediated endocytosis of some fungal elicitors (Horn *et al.* 1989). Once internalized, Avr/Pth proteins interact specifically with *R* gene products to form the dimers or multimers which are then transported into the nucleus of plant cells.

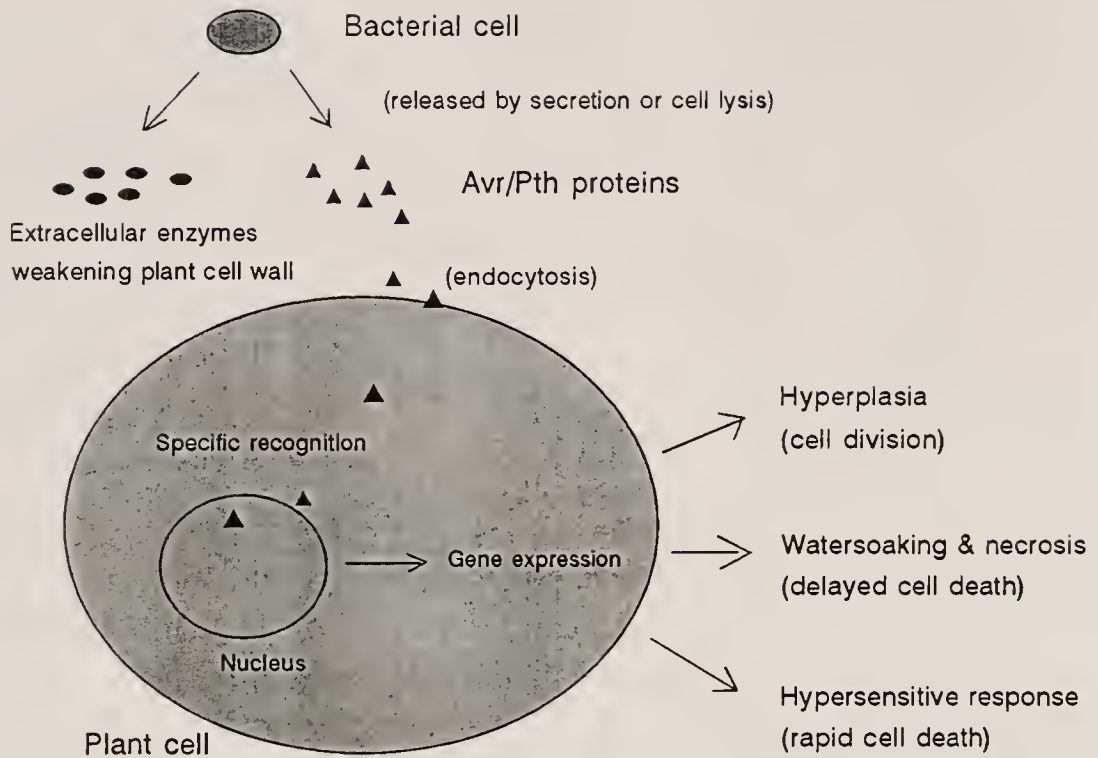


Figure 5-5. A working hypothesis for the transduction of *Xanthomonas* Avr/Pth signals during the plant-pathogen interaction.

Alternatively, Avr/Pth proteins are imported alone into the nucleus. The nuclear transport process can be activated or inhibited by many factors such as phosphorylation or dephosphorylation at specific casein kinase II sites of proteins (Rihs *et al.* 1989, 1991). After entering the nucleus, Avr/Pth proteins may act on nuclear transcriptional factors encoded by *R* genes or even directly on the *R* gene themselves. Mediated by the 34 amino acid tandem repeats, the avirulence and pathogenic specificity can be determined during the protein-protein or protein-DNA interaction. Activation of specific targeted genes may lead to different physiological outcomes such as hyperplasia (cell division) on citrus, hypersensitive response (rapid cell death) on cotton or citrus, watersoaking and subsequent necrosis on cotton (delayed cell death). This may be similar to oncogenesis (cell division) and apoptosis (cell death) of mammalian cells that can be altered by the ratio and interactions among transcription factors in specific cells (Martin *et al.* 1994).

This hypothesis may apply to other model systems of specific recognition such as the *Cladosporium fulvum* AVR4 protein containing potential NLSs. However, diverse signal transduction mechanisms may have been exploited by hosts and pathogens to determine the specific recognitions involving plant disease resistance and susceptibility. Although gene-for-gene interactions can appear to be similar genetically, they may be quite different in terms of biochemical mechanisms.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Members of a *Xanthomonas* avirulence (*avr*) gene family were shown to play an important role in determining host-specific pathogenic symptoms of cotton blight and citrus canker. This host-specific pathogenicity (*pth*) function may be critical in conditioning the host range of *Xanthomonas*. All cotton blight strains examined to date of *X. campestris* pv. *malvacearum* carry 4 to 12 DNA fragments that hybridize with members of this gene family. Twelve hybridizing fragments have now been isolated from a single *X. campestris* pv. *malvacearum* strain XcmH1005. Six of them (*avrB4*, *avrB6*, *avrB7*, *avrBIn*, *avrB101* and *avrB102*) are clustered on a 90-kb indigenous plasmid and were previously isolated as functional *avr* genes (De Feyter and Gabriel 1991a). The other six appear to be chromosomal fragments and were isolated in this study. One of these encodes the previously described *avrBn* (Gabriel *et al.* 1986), and three of these encode previously undescribed *avr* genes (*avrB5*, *avrB103*, and *avrB104*).

To investigate possible pleiotropic functions of these *avr* genes in XcmH1005, marker exchange mutagenesis and complementation analyses were carried out. A single mutation of *avrB6*, but not other members in XcmH1005, resulted in significant reduction of the pathogen's ability to induce watersoaking symptoms on cotton susceptible line Acala-44. An average of 240 times more bacteria were released onto the cotton leaf

surface from watersoaked spots caused by XcmH1005 than from those caused by an isogenic *avrb6*⁻ mutant. Another XcmH1005 mutant, in which seven *avr* genes were inactivated by repeated cycles of mutagenesis, was almost asymptomatic on cotton and had 1600 times less bacteria released onto the leaf surface in comparison with that of XcmH1005. However, *in planta* growth rates and yields of these mutants were identical to that of the parental XcmH1005 strain. Therefore, inactivation of multiple *avr* genes in *X. campestris* pv. *malvacearum* basically created a nonpathogenic endophyte of cotton. The pleiotropic pathogenicity function of these *avr* genes was further confirmed by complementation tests. Introduction of a DNA fragment carrying only *avrb6* into the *avrb6*⁻ mutant fully complemented the phenotypic defects for both avirulence and pathogenicity. Introduction of DNA fragments carrying *avrb6* plus two other *avr* genes into the latter mutant fully restored its pathogenicity, indicating an additive *pth* function of these *avr* genes. The role of these *avr* genes in eliciting host-specific symptoms and aiding pathogen release and dispersal strongly indicates their fitness value in cotton blight pathogen. The pleiotropic pathogenicity function and fitness value may partly explain why plant pathogens carry *avr* genes which usually act as negative factors to limit virulence on plants carrying specific *R* genes. However, the presence of many other *avr* genes in plant pathogens may simply results from mutations neutral to the selection.

In citrus canker disease, the host-specific canker symptoms are known to be determined by *pthA* of *X. citri*, another member of what is now known as the *avr/pth* gene family. Gene *pthA* also confers host specific pathogenicity and avirulence function to many xanthomonads on their respective hosts. DNA sequence comparisons revealed

that *pthA* is 98% identical to *avrBs3* and *avrBs3-2* of *X. campestris* pv. *vesicatoria*, 97% identical to *avrb6* of *X. campestris* pv. *malvacearum* and 95% identical to *avrXa10* of *X. oryzae* (Hopkins *et al.* 1992) along its entire length. The primary sequence differences among these genes lie in the central region of the genes, characterized by nearly identical, 102 bp tandem repeats. By constructing chimeric genes among *pthA*, *avrB4*, *avrb6*, *avrb7*, *avrBln*, *avrB101* and *avrB102*, the 102 bp tandem repeats were found to determine the gene-for-gene specificity of the avirulence reactions on cotton. In addition, the tandem repeats of *avrb6* and *pthA* determined their specificity in enhancing watersoaking on cotton and causing cankers on citrus, respectively. Replacement of tandem repeats of *avrb6* in XcmH1005 with those of *pthA* conferred to the cotton pathogen the ability to induce hyperplastic canker lesions on nonhost citrus at high inoculum level.

By inserting a *npt-sac* cartridge into the tandemly repeated region of *pthA* as a selective marker, intragenic recombination among homologous repeats was observed when the gene was present in both *Xanthomonas* and *Escherichia coli* strains. Intragenic recombination within *pthA* created new genes with novel host specificities and altered pathogenicity and/or avirulence phenotypes. Many *pthA* recombinants gained or lost avirulence function in many different combinations in pathogenicity assays on bean, citrus and cotton cultivars. Intragenic recombination therefore provides a genetic mechanism for the evolution of this *avr/pth* gene family.

It is striking that only a few amino acid changes in the tandem repeats of a single gene altered avirulence and pathogenic specificities. To account for the precise

recognition and diverse specificities, it is likely that these *avr/pth* genes encode protein elicitors capable of interacting with plant receptors and inducing host-specific symptoms. Unfortunately, elicitor activities encoded by *pthA* or *avrb6* were not detected despite repeated attempts. By analysis of the predicted amino acid sequences encoded by *pthA*, *avrb6*, and their homologues, however, three nuclear localization consensus sequences were found in the C-terminal region of these proteins. The predicted PthA and Avrb6 proteins were constitutively expressed in *Xanthomonas* as detected by Western analysis using anti-PthA antibody. By creating fusions of the C-terminus coding region of *pthA* and *avrb6* to a *gus* reporter gene and transiently expressing these fusion genes in onion epidermal cells, the C-terminal region of PthA or Avrb6 was observed to direct the GUS fusion protein into the nuclei. A working hypothesis is proposed that Avr/Pth proteins are secreted, taken up by the plant cell, enter the nucleus and directly affect gene expression, leading to the induction of host-specific symptoms.

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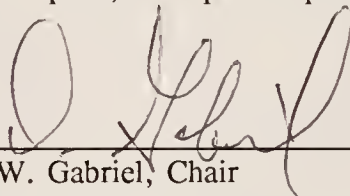
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BIOGRAPHICAL SKETCH


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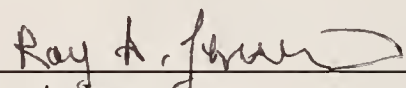
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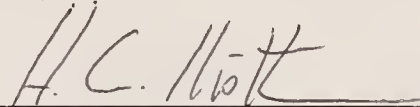
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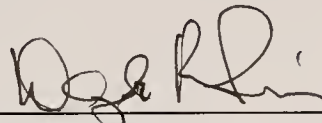
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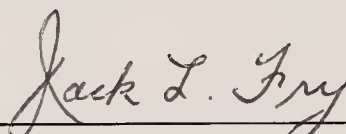
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1994



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